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THE ROLE OF ALKALINE PHOSPHATASE IN INTESTINAL ABSORPTION

I. THE KINETICS OF PHOSPHATASE ACTION ON VARIOUS SUBSTRATES¹

BY NEIL B. MADSEN AND JULES TUBA

Abstract

The kinetics of intestinal alkaline phosphatase action on sodium β -glycerophosphate, glucose 6-phosphate, and egg lecithin have been studied and compared. The Michaelis constants indicate that the enzyme shows considerably less affinity for lecithin than for the other two substrates, and the approximate ratio of activity with lecithin, glucose 6-phosphate, and sodium β -glycerophosphate is 11 : 78.5 : 100. The energies of activation for the hydrolysis of the three substrates do not differ appreciably and the average energy of activation is 14,500 calories per gram-mole. The similarity of the energies of activation together with results from inhibition studies indicate that in all probability the same enzyme is responsible for the release of inorganic phosphorus from each of the three substrates.

Introduction

The intestinal mucosa contains one of the highest concentrations of alkaline phosphatase of any tissue in the body (7, 12). Investigators have long surmised that this phosphatase is associated with the absorption of various foodstuffs in the small intestine. There is some evidence (3, 10, 22, 23) that glucose and possibly other hexoses are phosphorylated during their transport across the intestinal wall, and phosphatase would presumably act to dephosphorylate the hexose phosphates before their passage into the blood. The exact mechanism of sugar absorption is still to be elucidated, however. The mechanism of fat absorption is complicated and has been the subject of considerable investigation and debate. A survey of the literature is impossible here, but a recent short review by Frazer (8) covers the salient points. It is believed that phospholipids are formed in the intestine and facilitate fat absorption but that this mechanism may not be obligatory. Phosphatase might be expected to act at this stage in fat absorption.

Serum alkaline phosphatase levels in the rat have been shown to be correlated with food consumption (21), and in particular with the ingestion of fat (4).

¹ Manuscript received September 5, 1952.

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council, Ottawa, Canada.

Evidence has been presented by a number of workers to show that the major part of the alkaline phosphatase activity in rat serum is derived from the intestine (6, 11).

Weil and Russel (24), and, subsequently, Taylor, Madsen, and Tuba (20), showed that only the lipid portion of diets fed to rats was effective in raising serum alkaline phosphatase levels above the low fasting levels. The latter workers showed that both oleic and stearic acids were effective, whereas glycerol was not. Weil and Russell (24) had shown that the short chain fatty acids were not effective.

Phosphatase has been further implicated in the transport of fat across the intestinal wall by work which shows that a high fat diet produces higher levels of the enzyme in the intestine than do normal diets (11). Flock and Bollman (6) showed that feeding of fat to rats produced high levels of alkaline phosphatase in the intestinal lymph, whereas other foodstuffs did not have this effect.

The evidence for a role of phosphatase in the transport of foods across the intestinal wall is thus indirect, although plentiful and varied in character. It was decided to investigate the properties of intestinal alkaline phosphatase in an attempt to obtain direct evidence of its role in food absorption and to try to elucidate the mechanism of its action. The investigation was begun by studying the action of intestinal phosphatase on three substrates. Sodium β -glycerophosphate was used because it is one of the commonest substrates. Glucose 6-phosphate was chosen because it is the most likely intermediate in the absorption of glucose. Lecithin was included as a typical phospholipid and because it may be an intermediate in fat absorption.

Experimental

Adult male albino rats, weighing approximately 300 gm., were used as the source of the intestinal phosphatase. A rat was killed by decapitation and the first 10 cm. of the small intestine from the pylorus was immediately removed and chilled. After it was washed with ice water, the intestine was everted on a glass rod and the mucosa was scraped off and was homogenized with ice water in a glass homogenizer. The homogenate was made up to 250 ml. and stored at 5°C. for not longer than four days before use. Aliquots of the homogenates were centrifuged and the supernatants were used in the experiments.

Phosphatase activity was determined by the method of Shinowara, Jones, and Reinhart (19), as modified by Gould and Schwachman (9). The incubation mixture contained 0.0136 molar veronal as a buffer and 0.0067 molar magnesium sulphate, as an activator. The unit of phosphatase activity is defined, for the purposes of this paper, as equivalent to 1 mgm. of phosphorus as phosphate ion liberated during the incubation period from the substrate used. The enzyme activity is expressed as units per 100 ml. of the homogenate referred to above. The molarity of the substrates used in each experiment is indicated in the results.

The sodium salt of glucose 6-phosphate was prepared from the barium salt by mixing the dry barium salt with a stoichiometric amount of sodium carbonate, with the subsequent addition of water and then filtration of the resultant solution. The concentrations of the glucose 6-phosphate solutions and of the lecithin solutions were calculated by determination of the total organic phosphorus. The egg lecithin was supplied by Nutritional Biochemicals Corporation.

Results and Discussion

The pH optima were established for the action of intestinal phosphatase on the three substrates. The incubation period was one hour. The molarities of the three substrates were 0.0106, 0.0089, and 0.0054, respectively. The results are shown in Fig. 1. The pH values indicated in the figure are the averages during the incubation period. It is seen that the pH optimum for alkaline phosphatase when acting on sodium β -glycerophosphate is 9.1; for glucose 6-phosphate, it is 8.8; and for lecithin, it is 8.1. The pH optima for the hydrolysis of sodium β -glycerophosphate and glucose 6-phosphate are reasonably close together, but the optimum for the hydrolysis of lecithin

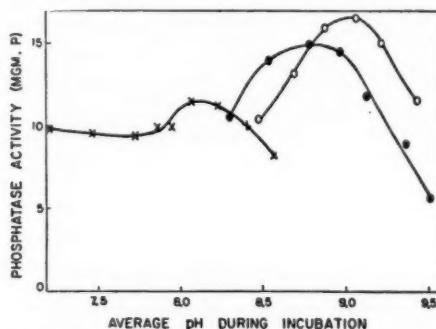


FIG. 1. The effect of pH on the activity of intestinal alkaline phosphatase with sodium β -glycerophosphate (O), glucose 6-phosphate (●), and lecithin (x). The values for the activity of the enzyme with lecithin have been multiplied by five.

is considerably different. This might raise a doubt as to whether the same enzyme is acting in each case. This point will be dealt with later. After the optimal concentrations of the substrates had been established (Fig. 3), the pH optima were redetermined but no observable change had occurred owing to the use of somewhat more concentrated substrates. The change in molarities of the substrates was probably not enough to produce the shift in pH optima reported for alkaline phosphatase by Ross, Ely, and Archer (15).

The relationship of time of incubation to phosphatase activity was then determined, and the optimal pH values for each substrate and the substrate

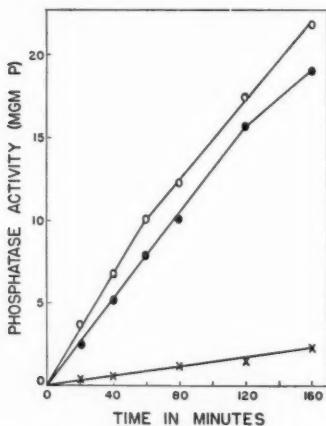


FIG. 2. The relationship of time of incubation to intestinal alkaline phosphatase activity with sodium β -glycerophosphate (○), glucose 6-phosphate (●), and lecithin (×).

molarities listed above were used. The results are depicted in Fig. 2. It was felt that the enzyme activity was constant for at least 60 min. with each substrate. The incubation period for subsequent work was kept within this time.

The effect of substrate concentration on the activity of alkaline phosphatase with the different substrates is illustrated in Fig. 3.

The value of the Michaelis constant, K_s , is calculated from the experimental data to be 0.00347 for sodium β -glycerophosphate, 0.00221 for glucose

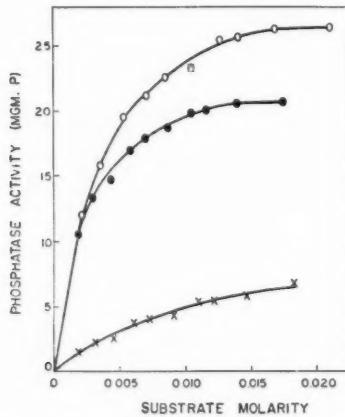


FIG. 3. The effect of substrate concentration on intestinal alkaline phosphatase activity with sodium β -glycerophosphate (○), glucose 6-phosphate (●), and lecithin (×). The time of incubation was 60 min. and the pH was optimal for the action of the enzyme on each substrate.

6-phosphate, and 0.0100 for lecithin. Intestinal alkaline phosphatase has therefore a much lower affinity for lecithin than for the other two substrates, and this may explain, in part at least, why the activity with lecithin is so much lower. The average activity of phosphatase with lecithin, for seven separate experiments, was $11.0\% \pm 1.3$ of the activity with sodium β -glycerophosphate; while the activity of the enzyme with glucose 6-phosphate was $78.5\% \pm 0.9$ of that with sodium β -glycerophosphate.

Delory and King (5) found that for a large variety of substrates there was a progressive increase in the rate of hydrolysis and the pH optimum and a progressive decrease in the K_s with decreasing pK of the substrate. Glucose 6-phosphate has a slightly lower pK than sodium β -glycerophosphate, and our finding that it also has a lower K_s could be expected on the basis of the results of Delory and King. In contrast to the expected results, however, the hydrolysis of glucose 6-phosphate by intestinal phosphatase is slower and occurs optimally at a lower pH than for sodium β -glycerophosphate. Since lecithin is a dipolar ion with an isoelectric point at pH 6.7, it is not possible to compare it with the other substrates on the above basis.

After examination of the curves in Fig. 3, new substrate concentrations were chosen in order to ensure that the enzyme would be fully saturated during the reaction time. The molarity chosen for sodium β -glycerophosphate was 0.0169; for glucose 6-phosphate, 0.0133; and for lecithin, 0.0137. These molarities were used in subsequent experiments, and also in checking the pH optima, as described above.

An attempt was then made to determine the energy of activation of the hydrolysis of each of the three substrates. Fig. 4 shows the plot of the natural logarithms of the activities at various temperatures against the reciprocals of the absolute temperatures used. The energies of activation were calculated from the data for the temperatures of 17° , 22° , and 27°C . by means

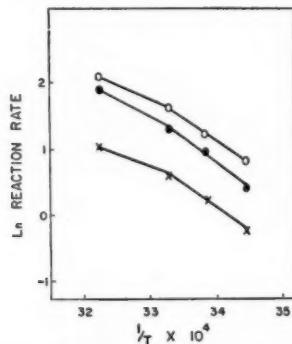


FIG. 4. The natural logarithm of the rate of hydrolysis of sodium β -glycerophosphate (O), glucose 6-phosphate (●), and lecithin (x) by intestinal alkaline phosphatase is plotted against the reciprocal of the absolute temperature ($1/T$).

of the formula, $\ln K = (EC/R) - (E^*/RT)$. The data obtained at 37°C. were excluded because there appears to be a slight inactivation of the enzyme at this temperature, despite the fact that the actual incubation time was only 20 min. in this experiment. This may be due to heat inactivation or inhibition of the enzyme by the greater concentration of inorganic phosphate at the higher temperatures (13, 14, 16).

The value of E obtained for the hydrolysis of sodium β -glycerophosphate was 13,900 cal. per gram-mole, while for glucose 6-phosphate E was 15,200 cal. and for lecithin E was 14,300 calories. The average energy of activation was $14,500 \pm 380$ cal. It is doubtful that the energies of activation determined here differ significantly from one another, and so it would appear that intestinal alkaline phosphatase has the same energy of activation for all three substrates. This would indicate that the same enzyme is acting on all three substrates. This energy of activation is considerably higher than the value of 9940 cal. determined for bone phosphatase by Bodansky (1). It is, perhaps, not surprising that bone and intestinal phosphatases should differ in their energies of activation, since they have been shown to differ in their Michaelis constants (2), and in their reaction to inhibitors (11).

The effect of inhibitors on the action of phosphatases on the three substrates is shown in Table I. The inhibitors were included in the usual substrate-buffer mixtures and the pH values were adjusted to give the optimal pH in the incubation mixtures. The inhibitors appear to have much the same effect on the activity of the enzyme with all three substrates. Oxalate reacted with lecithin and prevented the enzyme from acting on this substrate. Glutamic acid was also tried as an inhibitor, but it appeared to be bound by the lecithin and did not affect the phosphatase action. However, the effect

TABLE I
EFFECT OF INHIBITORS ON INTESTINAL ALKALINE PHOSPHATASE ACTIVITY
WITH VARIOUS SUBSTRATES

Inhibitor	Molarity	Residual phosphatase activity, % of original values		
		Sodium β -glycerophosphate	Glucose 6-phosphate	Lecithin
Sodium taurocholate	0.001	99.6	102.8	100.0
Sodium cyanide	0.01	1.6	1.3	0.0
Sodium oxalate	0.1	12.2	12.6	—†

* K = reaction rate; E = energy of activation in calories per gram-mole; C = an integration constant; R = the gas constant in calories; T = temperature in degrees absolute.

† A reaction occurs between sodium oxalate and lecithin and prevents a true measure of the actual inhibition due to the oxalate.

of the inhibitors used, together with the similar energies of activation, provide evidence that the same enzyme is liberating inorganic phosphorus from all three substrates.

Schmidt, Hecht, and Thannhauser (17) found that lecithin was degraded completely by rat intestine to fatty acids, glycerol, choline, and inorganic phosphate. If incubation was at pH 5.3 instead of at pH 7 to 8, there was an accumulation of glycerylphosphorylcholine. They believed that the latter substance is ordinarily hydrolyzed by the phosphatase of the intestine and that it is an intermediate of lecithin metabolism in the intestine. The purified intestinal phosphatase of Schmidt and Thannhauser (16) did not act on phospholipids but it did hydrolyze glycerylphosphorylcholine slowly (18). It is considered (18, 17) that the action of phosphatase on lecithin when crude extracts of the mucosa are used may depend on the prior action of specific "lecitholipases" to hydrolyze off the fatty acids and that the phosphatase may then act on the glycerylphosphorylcholine.

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THE RESPONSE OF CIRCULATING EOSINOPHILE CELLS TO MORPHINE AND RELATED SUBSTANCES¹

By J. C. SZERB²

Abstract

In order to investigate the possibility of adrenocorticoid hormones being released by narcotic drugs, the action of morphine, meperidine, and codeine on the number of circulating eosinophile cells was determined. In mice 10 mgm. per kgm. of morphine caused a significantly greater drop in the number of eosinophiles two hours after the injection than did 20 mgm. per kgm. of histamine. Adrenalectomy prevented the decrease following the injection of morphine and histamine. The comparison of the action of 10 mgm. per kgm. of morphine, 20 mgm. per kgm. of meperidine, and 30 mgm. per kgm. of codeine showed the greatest decrease in the number of eosinophiles after meperidine and the smallest after codeine. In humans 10 mgm. of morphine caused the largest (about 50%) decrease in the circulating eosinophiles followed by 50 mgm. of meperidine and 30 mgm. of codeine. The maximal fall occurred two hours after the injection. The possible mechanism of the action of narcotic drugs on the pituitary-adrenocortical system is discussed.

There is information in the literature showing a relationship between the action of morphine and the adrenal cortex. Thus MacKay (14) found an hypertrophy of the adrenal cortex after repeated injections of morphine in rats. Pucharich and Goetzl (17) and Friend and Harris (6) observed a decrease in the analgesic action of morphine after adrenalectomy. On the other hand adrenocorticotropin (ACTH) and cortisone showed effects which were similar to those produced by morphine. Patients who were treated with these hormones showed euphoria and a quick relief from pain, both of which could not be entirely ascribed to the sudden improvement in their condition (1, 8, 9, 19, 23). Analgesia in the form of an increase in reaction time following painful stimuli in mice treated with ACTH and cortisone has also been demonstrated (11, 24).

This similarity of action between morphine and the adrenocorticoid hormones posed the question as to whether some of the actions of morphine could be ascribed to the release of adrenocorticoid hormones. The first step in the investigation of this question was to determine whether morphine has a specific action on the pituitary-adrenocortical system as compared to a general stressing agent such as histamine.

The only data available on this subject were those of Zauder (26, 27). This author found an increase in the linolenic acid content of the brain in rats following the repeated administration of ACTH, cortical hormones, and morphine. Hypophysectomy prevented the increase in the linolenic acid content of the brain after morphine administration. These observations suggest that there is a release of adrenocorticoid hormones after repeated administration of morphine. The increase in the linolenic acid content of the brain is a rather insensitive test for measuring the output of adrenocorticoid hormones since the increase was found only after repeated injections of large doses of ACTH and cortical hormones.

¹ Manuscript received September 2, 1952.

Contribution from the Department of Pharmacology, Dalhousie University, Halifax, N.S.

² Assistant Professor of Pharmacology.

The aim of this work was to investigate the action of morphine and related substances on the pituitary-adrenocortical system using a more sensitive method: the decrease in the number of circulating eosinophile cells. This method according to Speirs and Meyer (22) demonstrates the action of as little as 1 μ gm. of Compound F.

The findings presented in this paper show that morphine and meperidine have a powerful stimulating action on the pituitary-adrenocortical system of the mouse and that this effect can also be demonstrated in man after the administration of therapeutic doses of these drugs.

Methods

Experiments with Mice

Male Swiss albino mice six to eight weeks old were used. Injections were made intraperitoneally and care was taken to excite the animals as little as possible. The volume injected was always 0.2 ml. per 20 gm. body weight. Drugs were dissolved in physiological saline solution and heated to body temperature before being injected. The counting of the eosinophile cells was made according to the method of Speirs and Meyer (21).

Injections of all the drugs used in one series were made at zero hour and, at the times indicated in the experimental results, counts were made on equal number of mice belonging to each drug group. Only one count was made on each mouse. This procedure was repeated several times.

Adrenalectomy was performed under pentobarbital anesthesia and 2-3 mgm. desoxycorticosterone crystals were implanted. Experiments were performed one week later.

In toxicity studies the drugs were given intraperitoneally in a volume of 0.2 ml. per 20 gm. body weight. LD₅₀ was calculated according to the procedure of Litchfield and Wilcoxon (12).

Human Experiments

Eight subjects, four males and four females between the ages of 20 and 32, were used. All were apparently in good health at the time of the experiment. Only one (N.E.H.) had a history of food-allergy and showed an initial eosinophile count between 375 and 660. The other initial counts were between 72 and 500.

Eosinophiles in the blood taken from the finger tips were counted according to the method of Speirs and Meyer (21). The first count was made about 9.30 a.m. and was then followed by the injection. All injections had a volume of 1 ml. and were given intramuscularly into the arm. The drugs were dissolved in 0.9% saline with the exception of meperidine which was given from the Demerol hydrochloride ampoules of Winthrop-Stearns Inc. The injections were not painful. Blood samples were taken one, two, three, and four and one-half hours after the injection. The timing of the last count was made to suit the convenience of the subjects.

The individuals used in the experiment had breakfast, 11 a.m. coffee, and lunch as usual; they were allowed to smoke and to perform their laboratory work. They stopped their usual activities only when some effect of the injected drug (dizziness, sleepiness, nausea) prevented them from working. The subjects were not informed of the nature of the injected drugs and knew only that no real harm would be caused them. The time interval between the injections was at least one week.

Results

The aim of the Series I experiments was to compare the pituitary-adreno-cortex stimulating action of morphine with a known and generally used stressing agent—histamine. Sayers and Sayers (20) showed that 5 mgm. per kgm. histamine causes a decrease in the ascorbic acid content of the adrenals in rats. This decrease is known to be accompanied by the release of adrenal corticoid hormones. Halpern and Benos (7) found a 62% fall in the number of eosinophile cells in rats three hours following the injection with 1 mgm. histamine per 150 gm. body weight. We wanted to compare the action of morphine with that of histamine in two ways: the relative decrease in the number of eosinophiles caused by the two agents and the speed with which the decrease occurs. As Speirs and Meyer (21) have shown that even a mild handling of mice causes a drop in the number of eosinophile cells, saline injections were also included in the experiment.

Fig. 1 shows the data obtained in these experiments. The results are given in absolute numbers of circulating eosinophile cells. The more generally used percentage form has not been employed, as only one count was made on each mouse and therefore the normal value for each mouse was not available. The results show that morphine has a greater and more rapid effect on the number of eosinophiles than either the control saline or histamine. The

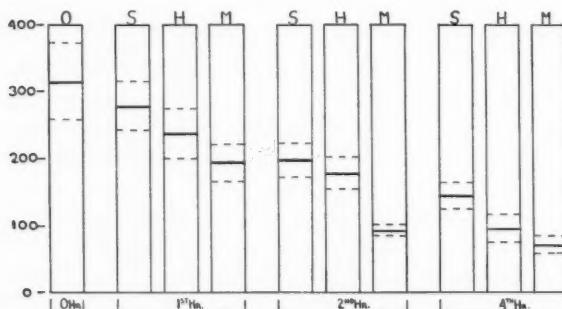


FIG. 1. The number of circulating eosinophile cells in untreated mice (0), and one, two, and four hours after the intraperitoneal injection of 10 ml. per kgm. of saline (S), 20 mgm. per kgm. of histamine biphosphate (H), and 10 mgm. per kgm. of morphine sulphate (M). The heavy line indicates the average. The area enclosed by the dotted line shows the plus-minus standard error of the mean. Each point was determined on 12 mice.

maximal drop caused by morphine is reached almost two hours after the injection and is significantly different from the saline group four hours after the injection. The histamine group does not differ significantly from those injected with saline. There is a considerable variation in the number of eosinophiles in normal mice and there is a significant fall in the eosinophile count after the injection of saline. These results are in good agreement with those of Speirs and Meyer (21).

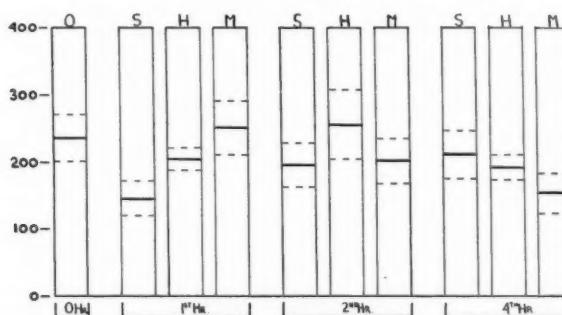


FIG. 2. The number of circulating eosinophile cells in adrenalectomized mice. The drugs injected and the symbols are the same as in Fig. 1. Each point was determined on 10 mice.

In Series II the experiments were performed in adrenalectomized mice. The same drugs were used as in Series I and counts were made in similar time intervals. Fig. 2 shows the results; it can be seen that no significant change occurred in the number of eosinophiles. This means that morphine as well as the other drugs injected act through the adrenal gland.

The experiments of Series III were designed to investigate further the specificity of the action of morphine and other related substances on the pituitary-adrenocortical system. Morphine and saline were used in the same way as in the preceding series, and the action of meperidine and codeine was investigated. Meperidine is not chemically related to morphine but has a similar action on pain sensitivity, and causes euphoria and eventually addiction. Codeine on the other hand, though chemically similar to morphine, has a much weaker analgesic action and does not produce euphoria and addiction. The use of these substances seemed to offer a chance to observe the correlation between the analgesic and euphoric action and the activation of the pituitary-adrenocortical system.

The problem of dosage arose in this experiment. It was thought that any substance in a sufficient toxic dose might activate the pituitary-adrenocortical system as a nonspecific stress. We determined the dose of meperidine which gave an analgesia comparable to the action of 10 mgm. per kgm. morphine, in mice, using the method of Jacob and Szerb (11). In Fig. 3 it can be seen that 20 mgm. per kgm. of meperidine causes a somewhat greater, but shorter

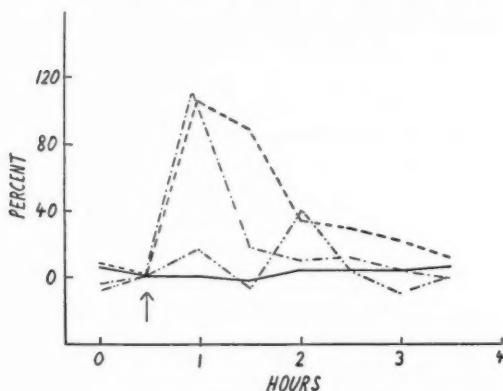


FIG. 3. Changes in reaction time following the intraperitoneal injection of 10 ml. per kgm. saline, 10 mgm. per kgm. morphine sulphate, 30 mgm. per kgm. codeine phosphate, and 20 mgm. per kgm. meperidine hydrochloride. The injections were made at the time indicated by the arrow. Each line shows the average of the results on 10 mice.
 — Saline. - - - Morphine. - · - - Meperidine. - · - - - Codeine.

lasting analgesia than does morphine. The determination of LD_{50} (Table I) showed that 20 mgm. per kgm. of meperidine is about one-seventh of the LD_{50} . Codeine however in a similar fraction of the LD_{50} caused only a small increase in the reaction time (Graph 3). Table I summarizes the LD_{50} of the drugs used in Series III as determined with mice of our colony and gives the fractions of LD_{50} used in this series.

Fig. 4 shows the results of the eosinophile counts in Series III experiments. It can be seen that the results obtained with saline and morphine are similar to those obtained in Series I. There is no significant difference between the codeine and the saline group nor between the morphine and the codeine group. Meperidine on the other hand shows a strong action two hours after the injection and the number of eosinophiles seems to increase four hours after the

TABLE I
 LD_{50} AND DOSES OF THE DRUGS USED IN SERIES III

Name of the drug	LD_{50}	Fiducial limits (P 0.05)		Doses used in Series III	Doses used in Series III as fraction of LD_{50}
		Upper	Lower		
Morphine sulphate	396.8	410.7	383.3	10	1/40
Codeine phosphate	212.8	226.0	200.3	30	1/7
Meperidine hydrochloride	155.6	164.6	147.1	20	1/7

NOTE: Doses are given in mgm. per kgm.

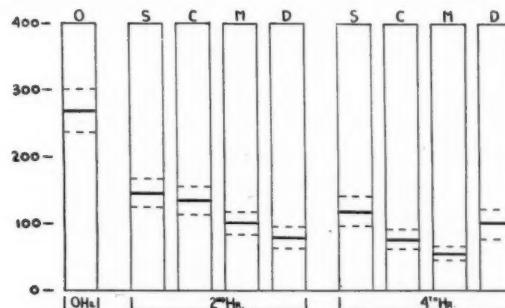


FIG. 4. The number of circulating eosinophile cells in untreated mice (0), and two and four hours after the intraperitoneal injection of 10 ml. per kgm. saline (S), 30 mgm. per kgm. codeine phosphate (C), 10 mgm. per kgm. morphine sulphate (M), and 20 mgm. per kgm. meperidine hydrochloride (D). The symbols are the same as those in Fig. 1. Each point was determined on 14 mice.

injection. This is similar to the very short analgesic action of meperidine. The rapidity of the action of meperidine was also observed by Radoff and Huggins (18).

This experiment shows further that there is a significant difference between the action of meperidine and codeine two hours after the injection though both of them were given in a dose which is about one-seventh of the LD_{50} . Morphine which was given in a dose only 1/40 of the LD_{50} has somewhat but not significantly stronger action than has one-seventh of LD_{50} of codeine.

Human Experiments

With the experiments on humans we wanted to determine whether therapeutic doses of morphine, meperidine, and codeine cause a stimulation of the adrenal cortex. The drugs were given in doses which are the lower limit of the therapeutic dosage, to avoid discomforting symptoms as much as possible. The results, which include those of the control saline group, are given in Figs. 5, 6, 7, and 8. The individual results in the saline group (Fig. 5) show certain variations from hour to hour but the average stays fairly constant and there is a slight increase in the average values. This is in agreement with the findings of Bonner (2) who found a steady level of eosinophile count in nonfasting individuals. Codeine (30 mgm.) caused an almost uniform but moderate fall two hours after the injection (Fig. 6). None of the changes approached the -50% level at this time. At the third hour the counts returned to normal. Meperidine (Fig. 7) had a more pronounced effect on four out of eight subjects whose eosinophile count went below -50% two hours after the injection. Two individuals reacted moderately and in two others there was no change. Morphine had the most consistent action as all seven persons injected had at least a 33% fall in their eosinophile number two hours after the injection and the average stayed low, to the end of the observation (Fig. 8). Statistical calculation shows that there is a highly significant difference between the effect of the three drugs and that

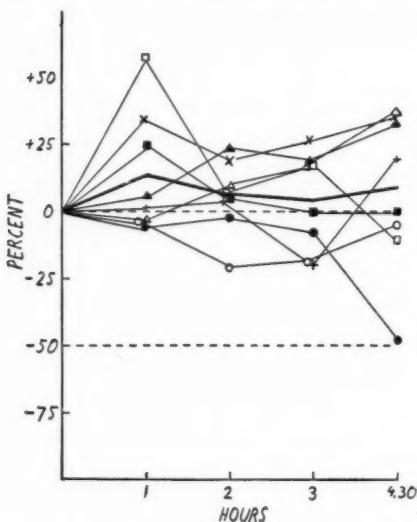


FIG. 5. Changes in the number of circulating eosinophile cells following the intramuscular injection of 1 ml. saline. □ J.S.T.; ■ E.H.A.; ▲ E.H.; △ N.R.B.; ○ N.E.H.; ● H.H.B.; + V.J.P.; × J.R. The first four individuals are males, the second four are females. The heavy line indicates the average.

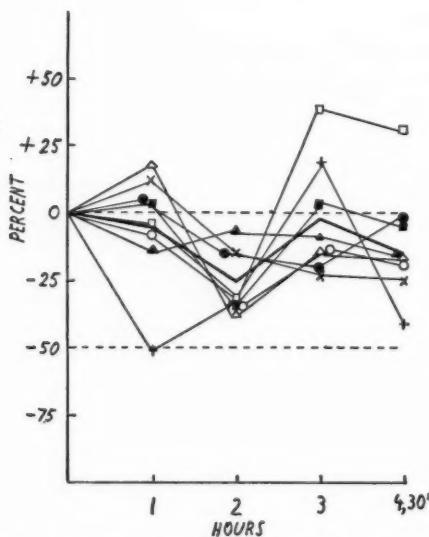


FIG. 6. Changes in the number of circulating eosinophile cells following the intramuscular injection of 30 mgm. of codeine phosphate. Symbols as in Fig. 5.

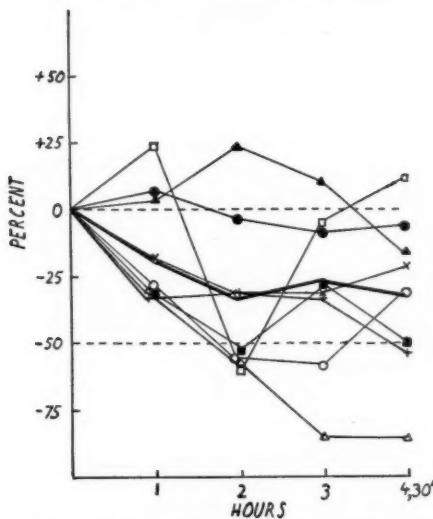


FIG. 7. Changes in the number of circulating eosinophile cells following the intra-muscular injection of 50 mgm. of meperidine hydrochloride. Symbols as in Fig. 5.

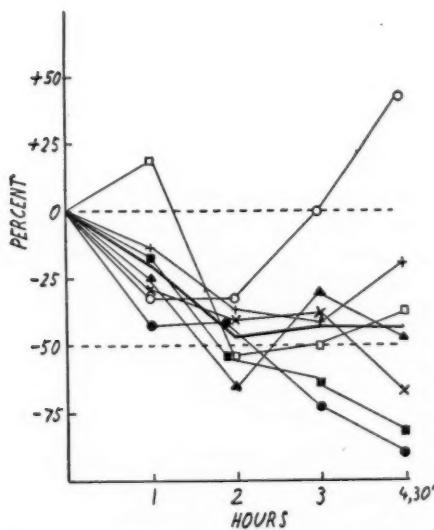


FIG. 8. Changes in the number of circulating eosinophile cells following the intra-muscular injection of 10 mgm. of morphine sulphate. Symbols as in Fig. 5.

of saline two hours after the injection. There is also a highly significant difference between the morphine and the codeine group two hours after the injection. The data of these experiments show further that in many instances the maximal drop was reached in two hours and the results were the most consistent at this time.

The correlation between the symptoms and the decrease of eosinophiles must be mentioned. The individuals whose count did not drop below -60% showed either a mild euphoria or did not experience anything. On the other hand in the cases of greater fall sleepiness or nausea developed to such an extent that they were unable to eat their lunch or continue their work. But in these cases the fall in the number of eosinophiles preceded the development of these symptoms by several hours. So a secondary action on the number of eosinophiles due to a general stress reaction caused by the symptoms following the injection can be excluded. Rather it might be supposed that the individuals who showed first an extreme decrease in eosinophiles and later the pronounced symptoms were more susceptible to the drug than were the others.

Discussion

The experimental results represented in this paper show that morphine and meperidine stimulate the pituitary-adrenocortical system. Their action seems to be different from that of a general stressing agent such as histamine. Their action is quicker and appears after the injection of a relatively small dose.

There are two probable ways by which morphine and meperidine could stimulate the secretion of adrenocorticoid hormones.

It is known that epinephrine is a potent stimulant of the secretion of the adrenal cortex (13, 15, 25). It is also known that morphine releases epinephrine from the adrenal medulla by central stimulation of the splanchnic nerves (5), and it is possible that the released epinephrine stimulates the secretion of the adrenocorticoid hormones.

The other way might be the direct stimulation of the hypothalamic centers by the narcotic drugs. It is known that morphine has a stimulant action on some of the hypothalamic centers, causing hyperglycaemia and stopping diuresis (3, 5, 6). On the other hand it has been shown that the electric stimulation of certain parts of the tuber cinereum and the mammalian bodies caused an activation of the pituitary-adrenocortical system (4, 10, 16). Lesions of these centers prevent the activation of the system by emotional stress. It might be that morphine and meperidine act through the stimulation of the hypothalamic center which is responsible for the activation of the pituitary-adrenocortical system. To decide this question further experimentation is necessary.

Acknowledgment

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LIVER GLUCURONIDASE ACTIVITY OF RATS IN VARIOUS EXPERIMENTAL STATES¹

BY JULES TUBA

Abstract

Liver glucuronidase activity was studied in male and female rats with regard to the effect of a number of experimental factors. Virgin female and breeder female rats had significantly higher levels of the liver enzyme than males. Breeding and lactation stimulated production of liver glucuronidase and activity fell towards male values in females which had passed the breeding age. Castration of males or females had no significant effect on the enzyme. A highly significant elevation of liver glucuronidase resulted from injection of oestradiol dipropionate into castrate male rats, while testosterone dipropionate had no effect. Neither hormone affected activity of the enzyme when injected into normal male or female rats. Levels of liver glucuronidase in diabetic male rats were elevated about 50% above normal, and these statistically significant increases are assumed to be related to some hormonal imbalance.

Introduction

Several papers from this laboratory indicate that age, sex, previous pregnancy, and a number of experimental factors can affect the levels of certain enzymes in tissues of laboratory animals. This has been found to be the case with respiratory enzymes of rat mammary tissue (10, 13), rat serum tributyrinase (11), rat serum alkaline phosphatase (1, 9), and mouse serum tributyrinase (8). These studies have now been extended to include rat liver glucuronidase. An investigation of this nature has been carried on with strains of inbred mice by Morrow, Greenspan, and Carroll, who have shown that differences exist in the levels of glucuronidase in the livers of various strains (5), and that activity of the enzyme in the kidneys of these animals is affected by sex (6).

Experimental

Rats of the Wistar strain were used in the investigation. They were generally housed in large cages in groups of six according to sex, age, and experimental state. The laboratory diet of Purina Fox Checkers and water was provided ad libitum.

Animals were killed by decapitation and livers were removed at once. A weighed portion of liver was ground in a chilled glass homogenizer, and glucuronidase levels were determined by the method of Talalay, Fishman, and Huggins (7). Glucuronidase activity is equivalent to the number of micrograms of phenolphthalein liberated by 1 mgm. of wet tissue in one hour at 37° C. from phenolphthalein mono- β -glucuronide at pH 4.5. The results are the averages of duplicate determinations.

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Results and Discussion

Normal Rats

The effects of age, sex, and previous pregnancy on rat liver glucuronidase are indicated in Table I. The statistical analyses show that the values are significantly affected by all these factors. There is no effect of age in males but there is a definite trend in females towards lower values with increasing age. In female rats over 50 weeks, when the animals are past the breeding age, the distinction between levels of liver glucuronidase in virgin and breeder females is no longer significant. Activity of the enzyme is greater in virgin female rats than in males and the breeders in turn possess greater liver glucuronidase activity than the virgins. The data of Table I suggest that levels of the enzyme are associated with female sex hormone activity.

TABLE I

LIVER GLUCURONIDASE ACTIVITY (UNITS PER MGM.) (G) IN MALE, VIRGIN FEMALE, AND BREEDER FEMALE RATS

(Numerical values are averaged and standard errors of means are indicated)

Age in weeks	Males		Virgin females		Breeder females	
	No. of rats	G	No. of rats	G	No. of rats	G
20	10	7.99 \pm 0.17	12	10.17 \pm 0.20		
30-40	17	7.62 \pm 0.13	6	7.29 \pm 0.38	13	11.91 \pm 0.14
Over 50	6	7.60 \pm 0.20	6	8.37 \pm 0.11	6	9.04 \pm 0.20
Total	33	7.73 \pm 0.04	24	9.01 \pm 0.12	19	11.00 \pm 0.15

Statistical analysis of data of Table I

Groups compared	Value of "t"	Value of "P"
20-week virgins vs. 30- to 40-week virgins	3.46	<0.01
20-week virgins vs. 50-week virgins	2.40	<0.05
30- to 40-week breeders vs. breeders over 50 weeks	3.45	<0.01
20-week males vs. 20-week virgins	3.25	<0.01
30- to 40-week males vs. 30- to 40-week breeders	4.25	<0.01
All males vs. all virgins	2.67	<0.01
All males vs. all breeders	5.73	<0.01
All virgins vs. all breeders	4.16	<0.01

Lactating Rats

Liver glucuronidase levels in nine lactating rats, 30 weeks of age, averaged 18.62 ± 1.80 units/mgm. and these were significantly higher than the average value of 12.12 ± 0.60 units/mgm. for nine nonlactating breeders of the same age. The value of t is 3.33 ($P = <0.01$).

Pregnant Rats

Seven animals chosen at random from the breeding cages, and in various stages of pregnancy, had liver glucuronidase levels varying from 6.80 to 12.43 units/mgm. The mean value of 9.96 ± 0.86 units/mgm. is lower than the normal average value of 12.12 ± 0.60 units/mgm. for nonpregnant breeders of the same age. Although the means are not significantly different, the range for the pregnant animals is somewhat lower than the range of 10.32 to 15.33 units/mgm. for the nonpregnant breeders.

It would appear that lactation, and perhaps pregnancy, associated with changes in female hormone secretion, are reflected in the activity of the enzyme.

Orchidectomized and Oophorectomized Rats

Values for the liver enzyme in seven 30-week old castrated male rats averaged 6.70 ± 0.53 units/mgm. and this is not significantly different from the average value of 8.10 ± 0.50 units/mgm. for 11 normal males of the same age.

The average activity of the enzyme in the livers of five 20-week-old castrated virgin female rats was 10.20 ± 1.2 units/mgm., which is not significantly different from the value of 10.17 ± 0.20 units/mgm. for 20-week old controls (Table I).

Responses to Injection of Sex Hormones

Groups of six adult animals were used to test the effect of injection of sex hormones on liver glucuronidase activity. Testosterone propionate (1.0 mgm. daily) or oestradiol dipropionate (0.1 mgm. daily), dissolved in oil, was injected subcutaneously into each animal for seven days.

The mean value of liver glucuronidase activity in castrate males receiving testosterone was 7.57 ± 0.26 units/mgm., which is not significantly different from control values of 8.10 ± 0.50 for normal males. Oestradiol in castrate males resulted in mean enzyme values for the group of 12.88 ± 1.30 units/mgm., which is significantly elevated above the control value ($t = 3.68$: $P = <0.01$).

The injection of testosterone and of oestradiol into groups of normal male rats resulted in mean enzyme values of 8.86 ± 0.69 and 7.55 ± 0.35 respectively, and these do not differ significantly from the control values. Similarly the effects of the two hormones on liver glucuronidase levels in normal breeder females were not significant.

Fishman and co-workers (2) have found that in intact or castrated mice of pure inbred strains the administration of estrogen, but not of androgen, resulted in increased activity of liver glucuronidase after 14 days. In our experiments with rats we were able to obtain elevation of liver glucuronidase activity with estrogen in castrate males only.

Alloxan Diabetic Rats

Adult male rats, 30 weeks of age, which had well established alloxan diabetes, were used in this experiment. The average liver glucuronidase activity for the 23 animals in this group was 12.40 ± 0.69 units/mgm. and this is about

50% higher than the average value of 8.1 ± 0.50 units/mgm. for 11 normal males of the same age. The difference between the two groups is highly significant ($t = 5.24$; $P = < 0.01$).

This increased concentration in liver enzyme can not be accounted for on the basis of altered weights of the livers, since these are approximately the same in diabetic and normal rats. It has been reported by Tuba and Madsen (12) and Tuba and Taylor (14) that the increased levels of serum alkaline phosphatase and of serum tributyrylase in alloxan diabetic rats show a highly significant correlation with increased food consumption. This explanation cannot, however, be used in the case of liver glucuronidase.

Lindan and Morgans (4) have observed that female rats with long standing alloxan diabetes manifested disturbances in oestrus and fertility. The decrease in fertility was accompanied by increased stillbirths and a greater number of neonatal deaths. Their findings were in all probability associated with profound disturbances in the hormone balance in the experimental animals. Abnormalities in hormone secretion might very well be associated with the very pronounced elevation of liver glucuronidase in our alloxan diabetic male rats.

Levvy and co-workers (3, 15) have produced evidence to support their claim that β -glucuronidase functions in the proliferation of tissue rather than in the synthesis of a steroid hormone glucuronides as proposed by Fishman (2). Our experiments with rats suggest that the effects on the liver enzyme of sex, age, breeding, lactation, and injection of estrogen into castrate males are associated with female hormones, which appear to stimulate glucuronidase production. On the basis of evidence available the elevated liver enzyme values in alloxan diabetic rats appear to be a reflection of hormone imbalance rather than tissue proliferation.

Acknowledgments

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THE EFFECTS OF DIET AND COLD ON BODY COMPOSITION AND FAT DISTRIBUTION IN THE WHITE RAT¹

BY EDOUARD PAGÉ AND LOUIS-MARIE BABINEAU

Abstract

From an insulation standpoint, high fat rations may be beneficial in the cold through an increase in total body fat but not through a more efficient distribution with respect to preservation of body heat since neither the fat content of the ration nor environmental temperature affect fat distribution. Any difference in efficiency of food utilization between high and low fat diets appears to be the same in the cold as at room temperature. Livers are smaller on a high fat ration both at room temperature and in the cold. Both livers and kidneys are larger in the cold than at room temperature. Average values for body water expressed as percentages of the lean body mass were the same in the cold as at room temperature.

Introduction

The beneficial effect of high fat rations in rats exposed to cold was first reported by Dugal, Leblond, and Thérien in 1945 (2). In 1946, Mitchell and co-workers (6) found that in man a high fat diet is superior to a high carbohydrate one in maintaining general psychomotor and visual performance in the cold. The cooling of internal tissues of the body on exposure to intense cold was greater on the high carbohydrate diet but only when the interval between meals is reduced to two hours. These authors are of the opinion that the superiority of high fat meals seems to be related to heat emission rather than heat production and that possibly after a high fat meal, fat is temporarily laid down in the subcutaneous tissues thereby affording some additional insulation.

The purpose of the first experiment described here was to study the effect of a high fat ration on fat distribution and caloric intake at room temperature and in the cold with a view to finding whether any demonstrable superiority of high fat diets could be ascribed either to better insulation or to a more efficient food utilization. In a subsequent study high fat rations with different protein contents were compared at both temperatures. We wondered whether increasing further the ketogenic properties of a high fat ration by raising its protein content at the expense of the carbohydrate component might be deleterious.

Experimental

High and Low Fat Rations

The composition of the rations used is shown in Table I; both had a "medium" protein content. Such a level, which amounts to 15% by weight of the low fat ration, was considered to be adequate at room temperature for rats of this size. The second experiment showed this assumption to be valid. In the cold, where food intake is nearly doubled, it follows that as

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much protein is ingested as would be the case for rats living at room temperature on a ration containing about 28% of protein, hence the term "medium" which we have applied to the protein level of these rations. This cutting back of the protein level from the usual 18 or 20% was done in order that protein intake in the cold would not be unduly high. The low fat ration contains 5% fat by weight, a level commonly used in rat experimentation though it is low by human standards. The protein, mineral, vitamin, and cellulose contents are the same in both rations on a calorie basis (using the arbitrary 4:4:9 ratio for calories per gram of protein, sugar, and fat, respectively). These adjustments were made at the expense of the sucrose.

Five groups of male rats were treated as follows. One control group of 20 rats was killed initially, all animals having been fed Purina Fox Chow up to that time. Two groups of 30 rats each were kept at room temperature and fed the high and the low fat rations, respectively. Two more groups, also of 30 rats, were fed these two rations, respectively, in the cold. They were exposed for the first 16 days to a temperature ranging between 6° and 10° C. and to a temperature of 3° C. ($\pm 2^\circ$) for the balance of the experimental period. They were sacrificed at the rate of six a day (three from each group) between the 117th and 127th day. Control rats kept at room temperature were killed immediately before or after the cold-exposed animals. This extended disposal period was made necessary by the number of different tissues dissected.

The rats were killed by decapitation under light ether anesthesia and no attempt was made to collect the blood lost. The perirenal fat (left side) was dissected and its fat content determined. This and the fresh weights of the liver, kidneys, and brown adipose tissue were the only measurements made on the control group killed initially. Changes in weights of the brown fat and perirenal fat have already been reported (8). In the four other groups, fat distribution was studied under the following headings: (a) *Subcutaneous fat*: the skin was dissected free of the underlying musculature and depot fat, weighed, and stretched on a board with a view to measuring pelt thickness. This did not prove feasible and the dried skin was later cut into thin strips and preserved in ethanol. (b) *Adipose tissue fat*: this fraction comprises the pelvic and scapular belts (panniculus adiposus) overlying the skinned carcass, the perirenal fat (which was actually determined separately), and the abdominal content including the testes and the emptied gastrointestinal tract but not the liver, spleen, and kidneys. (c) *Skeletal fat*: this fat was extracted from the remainder of the carcass including the liver, spleen and kidneys.

Fat extraction was carried out on the whole of each sample, which was preserved in alcohol prior to extraction, the carcass having been minced in a meat grinder and the depot fat cut into small pieces with scissors. Fat was first extracted in Soxhlet extractors with boiling alcohol (about eight hours) and then with ethyl ether until the residue assumed a white paperlike consistency.

The successive extracts of each sample were distilled to dryness *in vacuo*, taken up in petroleum ether, and combined. The petroleum ether extract

was made up to volume in the cold and total fat determinations were carried out on aliquots also measured in the cold. Where necessary, as in samples with a high water content, excess moisture was removed by distillation with an alcohol-benzene mixture, prior to the taking up of the lipids in petroleum ether.

Food consumption was measured during a period of 38 consecutive days for the rats kept in the cold and of 28 days for the others.

High Fat Rations at Different Protein Levels

The composition of these rations is shown in Table I, the medium protein one being identical to the one used previously. The fat, mineral, vitamin, and cellulose content was the same in both rations on a calorie basis. Five groups of rats were again used. One group of 20 was killed initially and the other four groups of 30 rats each were equally divided as to diet and environmental temperature. The experimental period was shorter (75 days) the rats having recovered their initial weight more quickly than in the previous experiment.

In the two groups kept in the cold room and in the initial group, determinations were made of the perirenal fat, total fat, and dry matter of the whole body. Fresh weights of the liver and kidneys were also recorded. Only perirenal fat and organ weights were measured in the control groups kept at room temperature.

The method of sacrificing the animals was modified so as to avoid loss of blood: the animals were weighed, killed by ether anesthesia, and the abdominal cavity was opened; the gastrointestinal tract was removed and rapidly emptied as follows: the caecum and stomach were removed, cut open, washed clean

TABLE I
COMPOSITION OF EXPERIMENTAL RATIONS

	Low fat	High fat	
	Medium protein	Medium protein	High protein
Casein	15.0	21.55	30.00
Cellulose (CellufLOUR)	1.0	1.44	1.44
Mineral salts	4.0	5.75	5.75
Vitamin mixture*	1.0	1.44	1.44
Sucrose	74.0	32.45	24.00
Crisco	2.5	25.00	25.00
Wheat germ oil	2.5	5.00	5.00
Mazola oil	0.0	10.00	10.00

* One gram of vitamin mixture in cellulose contains: thiamine hydrochloride, 0.4; riboflavin, 0.5; pyridoxine hydrochloride, 0.5; calcium pantothenate, 3.0; nicotinic acid, 3.0; inositol, 10; 2-methyl-1,4-naphthoquinone, 0.1; folic acid, 3.0; biotin, 0.2; choline chloride, 150 mgm. A supplement of vitamins A and D was given once weekly per os.

This corresponded to a dosage of about 200 I.U. of vitamin A and 35 I.U. of vitamin D per rat per day, irrespective of the diet.

with the help of a stream of water from a washing bottle, lightly blotted, and returned to the abdominal cavity. The small intestine was stretched on a damp board and its content squeezed out by pressing over the entire length with a small hand roller. The whole of each animal (including the emptied digestive tract) was then reweighed. The liver and kidneys were next taken out and weighed, care being taken to avoid any spilling of the blood. The whole carcasses were then placed in a cold room at -15° C. They were later ground in the frozen state and dried *in vacuo* at 50° C. for 36 hr. in an atmosphere of carbon dioxide for the determination of total dry matter. Fat extraction was then carried out as described previously. All nonfat residues from the various extractions were dried and combined. Their total weight plus that of the fat agreed well with total dry matter determined initially, indicating that any loss of material in the course of extraction was inconsequential.

Statistical Treatment

The fattening process is characterized by the preponderant deposition of fat in preferred regions, such as the abdominal cavity. As body fat increases, an increasing proportion of the total fat will therefore be found in such regions, while the proportion found in other fractions will suffer a corresponding decrease (although the fat content of each region actually increases). It follows that when comparing groups of different fat content, different proportions of the total fat may be found in a given region; this may simply indicate a normal and similar fat distribution, each group representing a separate point on the same fat distribution curve. In order to distinguish between a normal distribution curve and one that may have been perturbed by such factors as diet or environment, it is necessary to determine the straight or curvilinear regression lines for each set of values, the total fat being considered as the independent variable and the fat content of a given fraction as the dependent one. Although Brody (1) recommends using the parabola $Y = aX^b$ for relating part to whole, it was found in the present instance that such a log log relation yielded a practically straight line on arithmetic paper, within the spread of our data, b being almost equal to 1. At least as good a fit was obtained by the simpler straight line relationship $Y = a + bx$, as judged by the standard errors of the estimates and by comparing rho to r . Only the latter formula was used in the computations reported here.

Results

High Fat and Low Fat Rations

(a) Changes in Body Weight

Some rats died in the cold room and others were discarded from the control groups because of apparent ill health. They have been eliminated from all calculations of results. It is clear from Table II that the rats on a high fat ration made substantially larger gains in body weight at room temperature.

TABLE II

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON BODY WEIGHT CHANGES IN THE RAT

(Average initial body weight: 282 gm. for all groups)

	Number of rats	Final body weight (gm.)	Changes in body weight (gm.)	Significance of differences between groups (<i>P</i>)
Room temperature				
Low fat group	25	378	+ 96 ± 4.95	
High fat group	26	411	+ 129 ± 9.38	<.01
Cold room				
Low fat group	24	277	- 5 ± 5.05	
High fat group	24	295	+ 13 ± 4.16	<.01

$$* \text{Standard error: } \sqrt{\frac{\sum d^2}{n(n-1)}}.$$

The difference in body weight corresponds very nearly to the extra fat laid down (Table III). In the cold, the animals made little or no gain in body weight. They may, however, be considered as acclimatized in the sense that, by the end of the experiment, they had nearly recovered or exceeded their original body weight. It may be added that their physical appearance was exceptionally good and that very few had suffered any necrosis of the tail, a usually common occurrence. The rats on the high fat diet were significantly larger than the others and to that extent, our results confirm the earlier findings of Dugal *et al.* (2) as to the superiority of such rations in the cold.

(b) Fat Distribution

Table III shows the average fat content for all groups and its distribution in the three fractions described above. In rats kept at room temperature, the total fat content is significantly higher on a high fat ration and in these animals, a higher proportion of the total fat is found as adipose tissue fat and a lesser one as subcutaneous or skeletal fat, all differences being highly significant. Expressed as percentages of the body weight, skeletal and adipose tissue fat are higher on a high fat ration but there is no appreciable difference in skin fat. Finally fat concentration (in terms of fresh weight of tissue) is significantly higher only in the skeletal fat fraction.

The fat content of rats exposed to cold is only very slightly higher in rats on the high fat diet than in those on the low fat diet and not significantly so. No significant differences between these two groups occurred in respect of the various fat fractions.

TABLE III
EFFECTS OF DIET AND ENVIRONMENTAL TEMPERATURE ON FAT CONTENT AND DISTRIBUTION IN THE WHITE RAT

	Room temperature		Cold room		High fat
	Low fat	P	High fat	P	
Total fat					
Range (gm.)	29 - 86	< .01	40 - 159	.12 - .43	
Average (gm.)	57.91 ± 0.39	< .01	87.20 ± 4.98	29.89 ± 1.71	33.97 ± 1.46
% Body weight	15.44 ± 0.81	< .01	20.98 ± 0.88	10.69 ± 0.54	11.58 ± 0.52
Subcutaneous fat					
% Total fat	20.57 ± 1.11	< .01	16.14 ± 0.29	18.42 ± 0.66	> .1
% Body weight	3.16 ± 0.21	> .4	3.39 ± 0.18	2.00 ± 0.14	> .1
% Fresh weight of pelt	21.13 ± 1.20	> .1	23.40 ± 1.18	15.90 ± 1.02	> .3
Skeletal fat					
% Total fat	28.45 ± 1.00	< .01	25.34 ± 0.61	37.33 ± 1.25	> .1
% Body weight	4.27 ± 0.17	< .01	5.27 ± 0.21	3.87 ± 0.13	> .5
% Fresh weight of tissues	6.84 ± 0.28	< .01	8.79 ± 0.40	5.85 ± 0.22	> .2
Adipose tissue fat					
% Total fat	50.98 ± 1.17	< .01	58.46 ± .82	44.25 ± .99	> .4
% Body weight	8.01 ± .56	< .01	12.32 ± .60	4.88 ± .31	> .3
% Fresh weight of tissues	57.94 ± 6.08	< .2	67.61 ± 4.57	43.04 ± 5.81	> .6

TABLE IV

CORRELATIONS BETWEEN TOTAL BODY FAT AND SUBCUTANEOUS, SKELETAL, AND ADIPOSE TISSUE FAT FRACTIONS

		Coefficient of correlation (r)	Regression lines value of Y*	Standard error of the slope	Standard error of the estimate	
					Grams	%
Subcutaneous fat						
Room temp.:	Low fat	0.696 ± .16	0.196X + 0.59	± 0.04	3.22	26.94
	High fat	0.874 ± .09	0.170X - 0.71	± 0.02	2.35	16.69
Cold room:	Low fat	0.918 ± .09	0.235X - 1.39	± 0.02	0.81	14.48
	High fat	0.861 ± .11	0.213X - 0.53	± 0.03	0.99	14.76
Skeletal fat						
Room temp.:	Low fat	0.835 ± .12	0.155X + 6.93	± 0.02	1.62	10.21
	High fat	0.910 ± .09	0.192X + 5.01	± 0.02	2.17	10.00
Cold room:	Low fat	0.863 ± .11	0.214X + 4.36	± 0.03	1.01	9.38
	High fat	0.788 ± .13	0.220X + 4.22	± 0.04	1.21	10.31
Adipose tissue fat						
Room temp.:	Low fat	0.967 ± .06	0.649X - 7.51	± 0.04	2.74	9.10
	High fat	0.980 ± .04	0.639X - 4.38	± 0.03	3.26	6.35
Cold room:	Low fat	0.972 ± .05	0.551X - 2.97	± 0.03	1.05	7.79
	High fat	0.966 ± .05	0.568X - 3.72	± 0.03	1.07	6.89

* Y = Average value of a given fraction when the value for total fat is fixed at X.

The coefficients of correlation and linear regressions relating each fraction studied to total fat are shown in Table IV for all groups. The regression lines are further illustrated graphically in Figs. 1 and 2. When comparing two groups at the same temperature, no significant differences are to be found, although at room temperature there is a tendency for the high fat group to accumulate relatively more fat in the depot region and less in the skin. This is perhaps better seen in Fig. 3 where the distribution of fat has been translated into percentages of the total fat. Thus, when total fat amounts to 50 gm., 21% is found in the skin and 50% in adipose tissue fat for the low fat group, as compared to 16% and 55%, respectively, for the high fat group. Skeletal fat, on the other hand, shows identical changes in both groups with increasing fat content. These results in so far as they can be interpreted do not lend support to the assumption of Mitchell *et al.* (6) that a high fat diet favors fat deposition in the subcutaneous tissues. Any "excess fat" tends to be stored in adipose tissue proper.

It may be of interest to point out that with the exception of the lower values for the high fat group in the cold, subcutaneous fat represents an almost constant percentage of total fat irrespective of the amount of body fat (Figs. 3 and 4).

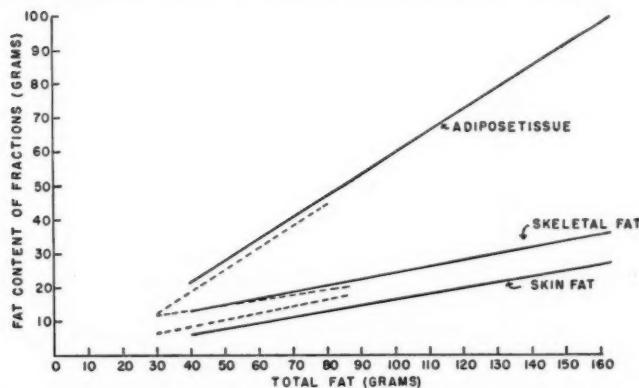


FIG. 1. Fat distribution at room temperature. Low fat -----, High fat ——.

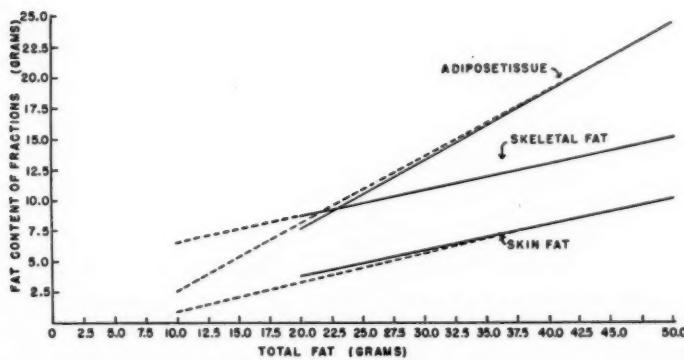


FIG. 2. Fat distribution in a cold environment. Low fat -----, High fat ——.

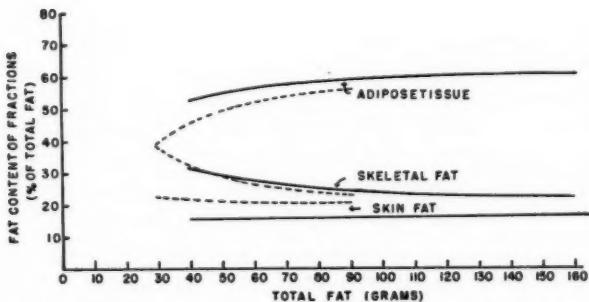


FIG. 3. Fat distribution as percentage of total fat at room temperature. Low fat -----, High fat ——.

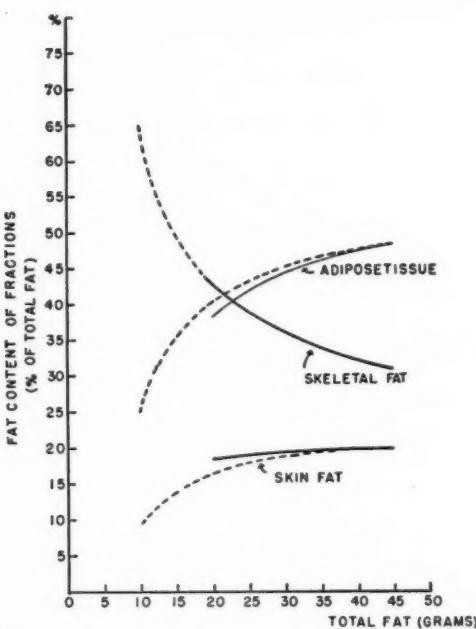


FIG. 4. Fat distribution as percentage of total fat in a cold environment. Low fat -----, High fat —————.

In the cold, fat distribution is identical in both groups, almost surprisingly so if one considers the experimental errors inherent to such small samplings. It may be safely concluded that in the cold, at least, the fat level of the diet has no influence on fat distribution (Figs. 2 and 4).

Whether exposure to cold influences fat distribution, irrespective of diet, cannot be answered with certainty since the control rats were much larger and little overlapping in fat content occurred. It may be significant, however, that where fat contents do overlap, e.g., at 40 gm. of total fat, values of 8.0, 13.0, and 19.0 gm. are found for subcutaneous, skeletal, and adipose tissue fat, respectively, for both groups in the cold, as compared to 8.5, 13.0, and 18.5 gm., respectively, for the low fat group at room temperature and 6.0, 13.0, and 21.0 gm., respectively, for the high fat group. It would seem, therefore, by comparison of rats exposed to cold with rats fed a low fat ration at room temperature, that exposure to cold does not affect fat distribution. Under our experimental conditions, the effects of cold are limited to a restriction of body growth and total fat content so that we do not encounter the obese animals which characterized the group fed a high fat ration at room temperature.

TABLE V
CORRELATION BETWEEN PERIRENAL FAT AND TOTAL BODY FAT

	Total fat content (range in grams)	Coefficient of correlation (r)	Regression lines values of Y^*	Standard error of the slope	Standard error of the estimate	Grams	%**
Room temperature							
Control group killed initially (2nd expt.)	17 - 42	0.812 \pm .14	10.69 X + 13.99	\pm 1.811	3.70	13.95	
Low fat group	29 - 86	0.854 \pm .11	10.26 X + 18.09	\pm 1.367	9.28	16.03	
High fat group	40 - 159	0.883 \pm .10	10.20 X + 17.87	\pm 1.108	11.69	13.40	
Three groups combined	17 - 159	0.96 \pm .04	10.77 X + 15.11	\pm 0.40	8.88	14.80	
Cold room							
Low fat group	12 - 43	0.921 \pm .09	11.85 X + 13.53	\pm 1.09	3.12	10.45	
High fat group	23 - 47	0.834 \pm .11	8.99 X + 19.14	\pm 1.27	3.87	11.41	

* Y = Values for total fat when the value for perirenal fat is fixed at X .

** Per cent of mean value for total fat.

Perirenal fat:—The relation between perirenal fat (left side) and total body fat is shown in Table V for the four groups fed the experimental rations and for the control group killed at the beginning of the following experiment. The regression line for the three groups kept at room temperature is further shown in Fig. 5. It would appear that under normal circumstances, the perirenal fat is a reliable index of total fat over a wide range of values. The relationship in rats living in the cold is slightly but not significantly different.

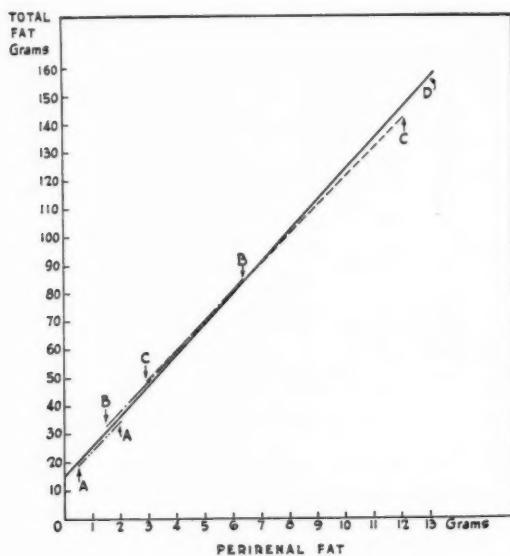


FIG. 5. Correlation between perirenal fat and total fat. AA = Initial control group; BB = Low fat group; CC = High fat group. D = Three groups combined.

(c) *Caloric Intake*

The caloric intake over part of the experimental period is shown in Table VI. A recent experiment in which these rations were pair-fed to rats indicates that the high fat ration has a higher fuel value than was anticipated from the arbitrary calculation of its caloric content. As a consequence, little significance can be attached to the magnitude of the difference in caloric intake between groups at a given temperature. It is to be noted, however, that this difference, on a percentage basis, is the same at both temperatures and that the superiority of one ration over the other must be the same in the cold as at room temperature.

TABLE VI
CALORIC INTAKE ON DIFFERENT DIETS AND AT DIFFERENT TEMPERATURES

	Room temperature			Cold room		
	Low fat	P	High fat	Low fat	P	High fat
Period of measurement (days)	28		28	38		38
Average body weight (grams)	356		378	277		291
Average gain in body weight (grams)	21		21	0		1
Caloric intake						
Cal. per day	63.0 ± .97	>.6	62.1 ± 1.97	102 ± 1.42	>.3	100 ± 1.74
Cal./100 gm. B.W./day	17.8 ± .91	=.2	16.5 ± .39	36.9 ± .70	<.01	34.3 ± .45
Cal./m. ² /day	1480 ± 18	=.05	1404 ± .34	2782 ± .41	<.01	2641 ± .35
Increase in caloric intake on low fat ration (%)	5.4			5.3		
Increased intake in the cold (%)				88		88

TABLE VII
LIVER AND KIDNEY WEIGHTS IN RELATION TO DIET AND ENVIRONMENTAL TEMPERATURE

	Initial control group	Room temperature			Cold room	
		Low fat	P	High fat	Low fat	P
Livers						
Fresh weight (gm.)	9.60 ± .34	13.69 ± .33	> .3	13.15 ± .40	13.13 ± .29	> .3
% Total body wt.	3.39 ± .09	3.68 ± .06	< .01	3.19 ± .05	4.73 ± .09	< .01
% Fat-free body wt.	3.68*	4.35 ± .08	< .01	4.05 ± .08	5.30 ± .10	< .01
Kidneys						
Fresh weight (gm.)	1.82 ± .06	2.17 ± .06	> .6	2.22 ± .08	2.34 ± .06	> .4
% Total body wt.	0.64 ± .02	0.58 ± .01	< .01	0.54 ± .01	0.84 ± .02	< .02
% Fat-free body wt.	0.70*	0.69 ± .02	> .7	0.68 ± .02	0.94 ± .02	< .02

* Total body fat estimated from perirenal fat (Fig. 5).

(d) *Organ Weights*

These are shown in Table VII. Expressing results as percentages of the lean body mass is particularly indicated when dealing with very fat animals since erroneously low values may be obtained if the results are based on total body weight. It is seen that livers are markedly larger in rats on the low fat ration at both temperatures. They are also larger in the cold, irrespective of the diet.

At room temperature, the level of fat of the ration does not affect the weight of the kidneys. In fact, the weights of the kidneys represent a remarkably constant percentage of the fat free body weight for all three groups at room temperature. Kidneys are larger in the cold in both groups and they are significantly larger on the low fat than on the high fat ration. These differences are probably related to higher caloric intakes in the cold as it affects likewise protein intake and catabolism.

TABLE VIII

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON BODY WEIGHT CHANGES IN THE RAT
(Average initial body weight: 295 gm. for all groups)

	Number of rats	Final body weight (gm.)	Changes in body weight (gm.)	Significance of differences between groups (P)
Room temperature				
Medium protein	29	440 \pm 13.4	145 \pm 7.1	$> .6$
High protein	29	436 \pm 9.0	141 \pm 7.2	
Cold room				
Medium protein	24	290 \pm 7.9	-5.5 \pm 4.4	$> .4$
High protein	20	295 \pm 7.0	-1.0 \pm 4.0	

High Fat Rations at Different Protein Levels

(a) *Changes in Body Weight*

Modification of the protein level of the ration had no particular effect on changes in body weight either in the cold or at room temperature (Table VIII).

(b) *Body Composition*

Body composition in terms of water, fat-free dry matter, and fat is shown in Table IX. There are no significant differences between groups although, as one might expect, water represents a lower percentage of total body weight when the fat content is higher (controls and high protein group). In the first experiment and on the basis of increases in the amount of perirenal fat in the cold, we had concluded that in the cold-adapted animals, some of the body

TABLE IX
BODY COMPOSITION OF RATS KEPT IN THE COLD AND OF CONTROL ANIMALS SACRIFICED INITIALLY

	Water content			Fat-free dry matter			Total fat	
	Grams	% Body weight	% Fat-free weight	Grams	%	Grams	%	
Control group	178.6 ± 4.00	64.44 ± .39	71.23 ± .33	70.61 ± 1.38	25.54 ± .33	26.5 ± 4.60	9.54 ± .44	
Medium protein	184.9 ± 5.11	65.47 ± .34	71.60 ± .41	73.71 ± 2.29	26.06 ± .33	24.1 ± 1.58	8.53 ± .50	
High protein	185.8 ± 5.01	64.45 ± .48	71.09 ± .28	76.32 ± 2.36	26.47 ± .35	26.6 ± 1.67	9.35 ± .67	

TABLE X
CORRELATION BETWEEN TOTAL BODY WATER AND FAT-FREE BODY WEIGHT

	X = Fat-free body weight		Standard error of the slope	Y = Total body water	
	Correlation coefficient "r"	Regression line value of Y		Standard error of the estimate	
				In grams	In % of the mean
Initial control group					
	0.984 ± .04	0.818X - 26.07	± .035	3.12	1.75
Cold room, high fat:					
Medium protein	0.976 ± .05	0.682X + 8.60	± .033	5.37	2.08
High protein	0.991 ± .03	0.678X + 8.52	± .021	2.91	1.56

water had been replaced by fat since these animals weighed less, or only slightly more, than the controls (8). This assumption is not invalidated by the present findings but it cannot be confirmed since no additional deposition of fat occurred in the course of exposure to cold.

Water represents 71% of the fat-free body weight in all groups, a value similar to that reported for man by Robinson and McCance (10) and slightly lower than that found by others for small animals (7). This relation is further analyzed in Table X. It may be noted that the relation is very strict as judged by the standard errors and the correlation coefficients. The regression coefficient for the control group differs significantly from that found for the other groups and in the sense that at room temperature the percentage of water increases with increasing body weight whereas in the cold, the opposite holds. The differences, however, are small and interpretation is complicated by the fact that regression coefficients reported by other authors (7) lie intermediate between those found here for controls and cold-adapted animals, respectively.

(c) *Organ Weights*

These are shown in Table XI. On the basis of the fat-free body weight, liver and kidney weights are comparable with those found in the preceding experiment for rats on the same experimental rations. As previously found, livers and kidneys are relatively larger in the cold than at room temperature. The protein level of the ration had no significant effect on the size of the liver at either temperature and on that of the kidneys at room temperature. The higher protein intakes in the cold resulted in significantly larger kidneys in the high protein group.

The livers of the control group are much larger than those of the control group in the previous experiment and, as a consequence, exposure to cold

TABLE XI
LIVER AND KIDNEY WEIGHTS IN RELATION TO DIET AND ENVIRONMENTAL TEMPERATURE

	Initial control group	Room temperature		Cold room	
		Medium protein	High protein	Medium protein	High protein
Livers					
Fresh weights (gm.)					
P** vs. controls	12.30 ± .40	13.71 ± .41	15.56 ± .28	11.62 ± .45	11.72 ± .38
% Total body weight	4.22 ± .08	3.14 ± .06	3.11 ± .06	4.00 ± .09	3.97 ± .07
P** vs. controls		<.01	<.01	>.05	<.02
% Fat-free body weight	4.63 ± .10	3.96*	3.76*	4.36 ± .10	4.37 ± .08
P** vs. controls				>.05	<.05
Kidneys					
Fresh weights (gm.)					
P** vs. controls	1.98 ± .06	2.27 ± .06	2.42 ± .08	2.31 ± .08	2.47 ± .09
% Total body weight	0.68 ± .02	0.52 ± .02	0.56 ± .02	0.80 ± .02	0.84 ± .02
P** vs. controls		<.01	<.01	<.01	<.01
% Fat-free body weight	0.75 ± .02	0.66*	0.67*	0.87 ± .03	0.92 ± .03
P** vs. controls				<.01	<.01

* Total body fat estimated from perirenal fat (Fig. 5).

** Probability of identity with initial control group.

results in an apparent decrease in the size of this organ. We cannot account for this except by the fact that the animals were bled to death in the first experiment and not in the other. This might explain the larger weight of the fresh liver of the control group in the latter case but a similar congestion should have occurred in the other groups killed similarly.

Discussion and Conclusions

As early as 1930, Reed *et al.* (9) had concluded that neither the nature of the diet nor exercise affects fat distribution in the white rat. More recently, Williams *et al.* (11) have reported that feeding a high fat diet increases the body content in neutral fat.

The results obtained indicate that the pattern of fat distribution with increasing fat content is not disturbed by environmental temperature nor by the fat content of the ration. As a consequence, any beneficial effect of a high fat ration on thermal insulation must be limited to an increase in the total fat stores. Such an increase did not materialize in the animals exposed to cold and fed a high fat ration and it must be concluded that under our experimental conditions, other factors than insulation were responsible for the observed superiority of the high fat ration. It should be emphasized that larger gains in body weight were our only criterion in assessing the respective values of the rations used and that it may not be the best one.

Forbes *et al.* (4) have reported that high fat rations are more efficiently utilized than low fat ones by mature animals at room temperature and *at rest*. Our own results, though resting on a far less accurate determination of energy expenditure, are in accord with these findings. These authors stress that this superiority of high fat rations need not enter into conflict with the numerous findings of other authors that work is performed more efficiently at the expense of carbohydrates than of fat. It is interesting that in the cold where energy expenditure was nearly doubled, the high fat ration registered the same superiority as at room temperature. This would probably not have been the case, were extra heat production in the cold due to muscular activity and its preferential demand on carbohydrates.

Emery *et al.* (3) have reported that exposure to cold causes a hypertrophy of the livers and kidneys. Our own findings confirm this.

Livers were generally larger on the low fat diet at either temperature and larger in the cold on both diets than at room temperature. If one accepts the general concept that organ size is related to organ work, it would follow that on the low fat diet, more work is done by the liver, possibly in connection with fat synthesis. Forbes *et al.* (4) state that the higher energy expense of food utilization with high carbohydrate diets resides precisely in the synthesis of fat from carbohydrates. It would thus appear that in spite of the higher energy expenditure resulting from exposure to cold, such a conversion of sugar into fat continues unabated. This view, though highly speculative on the basis of our data, is in accord with the findings of Kayser (5) that lipids are used preferentially for heat production in the cold.

Changes in the protein level of the ration from an adequate to a generous level did not affect the rats adversely (or otherwise) although the carbohydrate fraction of the diet was thereby seriously diminished. It is realized, however, that rats are much less susceptible than man to ketogenic diets.

The water content of the body on a fat-free basis does not seem to be affected by cold although modification in the cold of the regression coefficients defining this relation forbids any definitive conclusion.

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SERUM ALKALINE PHOSPHATASE LEVELS IN MICE OF THE C₅₇, C₃H, AND A STRAINS¹

BY JULES TUBA

Abstract

Levels of serum alkaline phosphatase have been established for mice of the C₅₇, A, and C₃H strains. Statistically significant effects on the activity of the enzyme are associated with age, sex, previous pregnancy, and strain. There is no evidence of correlation between enzyme values and resistance or susceptibility to cancer.

Introduction

Previous reports from this laboratory have shown that the sex and age of rats may have significant effects on the levels of serum alkaline phosphatase (5), serum tributyrinase (7), and of the mammary cytochrome system (6). Statistically significant differences have been demonstrated in the oxygen uptake by mammary tissue of virgin and breeder female rats (6). The investigation of the effect of age, sex, and previous pregnancy on various enzyme systems was extended to include a low-cancer strain of mouse, C₅₇, and two cancer-susceptible strains, C₃H and A. A report has been published on the levels of serum tributyrinase in these three strains of mice (4). It was shown that age, sex, previous pregnancy, and strain were associated with statistically significant variations in the activity of serum tributyrinase, but there was no evidence that this enzyme was related to susceptibility or resistance to cancer. The study of the influence of these four factors in the above three strains of mice included serum alkaline phosphatase, and the findings are reported here.

Experimental

Mice of three representative age groups were used (see Table I). They were housed in groups of approximately six in metal cages and given Purina Fox Checkers and water ad libitum. Male and virgin female mice were kept segregated throughout the experiment. Breeder females were segregated at least four weeks after the last litter to eliminate any effect of lactation on the activity of serum alkaline phosphatase. There were 150 C₃H, 103 C₅₇, and 97 A mice used in the investigation.

The animals were killed by decapitation and the blood was collected in 2-ml. conical centrifuge tubes. Immediately after it was separated, the serum was stored at 4° C. until alkaline phosphatase activity was determined. Storage was never for longer than three days and during this time the enzyme was found to be stable.

Serum alkaline phosphatase was estimated by the micromethod of Shinozawa, Jones, and Reinhart (3), as modified by Gould and Schwachman (1).

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TABLE I
SERUM ALKALINE PHOSPHATASE LEVELS IN MICE OF THE C₃H, A, AND C₅₇ STRAINS

Males				Breeder females				Virgin females			
Age (weeks)	No. of mice	Phosphatase (units/100 ml.)		Age (weeks)	No. of mice	Phosphatase (units/100 ml.)		Age (weeks)	No. of mice	Phosphatase (units/100 ml.)	
		Range	Mean			Range	Mean			Range	Mean
<i>C₃H strain</i>											
12-20	4	4.7-9.1	6.9	30-40	39	2.6-7.7	5.0	12-20	12	5.4-9.6	7.0
30-40	56	2.4-8.0	4.7	50-60	7	2.1-5.9	4.1	30-40	24	3.3-9.4	5.5
50-60	4	4.4-4.8	4.6	Total	46	2.1-7.7	4.9±0.28	50-60	4	3.5-5.6	4.8
Total	64	2.4-9.1	4.9±0.18					Total	40	3.3-9.6	5.7±0.24
<i>A strain</i>											
12-20	10	3.8-8.9	6.0	30-40	14	1.3-5.2	3.3	12-20	12	5.2-7.6	7.6
30-40	29	2.0-7.1	3.7	50-60	5	1.2-5.8	3.4	30-40	6	2.8-5.9	4.3
50-60	21	1.6-5.9	3.5	Total	19	1.2-5.8	3.3±0.30	50-60	18	2.8-7.6	6.5±0.40
Total	60	1.6-8.9	4.0±0.20					Total			
<i>C₅₇ strain</i>											
12-20	10	3.7-7.5	6.1	30-40	7	3.5-5.9	4.6	12-20	17	7.5-12.2	9.4
30-40	14	4.1-8.9	6.0	50-60	15	3.2-8.1	5.1	30-40	12	3.8-6.3	5.3
50-60	18	1.7-5.0	3.5	Total	22	3.2-8.1	5.0±0.32	50-60	10	2.1-4.9	3.7
Total	42	1.7-8.9	5.0±0.27					Total	39	2.1-12.2	6.7±0.45

The unit of phosphatase activity has been defined by Shinowara as "equivalent to 1 mgm. of phosphorus liberated as phosphate ion during one hour of incubation at 37° C., with a substrate containing sodium β -glycerophosphate, hydrolysis not exceeding 10% of the substrate and optimum pH of the reaction mixture for the alkaline enzyme at 9.3 \pm 0.15."

Results and Discussion

Serum alkaline phosphatase values are indicated in Table I for three age groups in each strain. These groups were: young adults of approximately 20 weeks, middle-aged mice of 30-40 weeks, and old mice of 50-60 weeks. The results are also grouped for males, virgin females, and breeder females. The standard errors of the means are given for the total number of males, breeders, and virgins of each strain.

Table I includes a statistical analysis of the mean serum alkaline phosphatase values. The data have been compared for differences which might be due to age, sex, or strain. Statistically significant differences are indicated, and in each case the value of t is given as well as the value of P . A probability of 0.05 or less is regarded as significant, while a probability of 0.01 is highly significant.

STATISTICAL ANALYSIS OF DATA OF TABLE I

Strain	Groups compared	Value of " t "	Value of " P "
<i>Age differences</i>			
C ₅₇ H	12-20 week males vs. 50-60 week males	2.46	<0.05
C ₅₇ H	12-20 week virgins vs. 50-60 week virgins	3.60	<0.01
A	12-20 week males vs. 50-60 week males	3.98	<0.01
A	12-20 week virgins vs. 50-60 week virgins	5.29	<0.01
C ₅₇	12-20 week males vs. 50-60 week males	4.66	<0.01
C ₅₇	12-20 week virgins vs. 50-60 week virgins	11.70	<0.01
<i>Sex differences</i>			
C ₅₇ H	All males vs. all virgins	3.33	<0.01
C ₅₇ H	All breeders vs. all virgins	3.37	<0.01
A	12-20 week males vs. 12-20 week virgins	2.47	<0.05
A	All males vs. all breeders	2.02	<0.05
A	All males vs. all virgins	5.04	<0.01
A	All breeders vs. all virgins	5.92	<0.01
C ₅₇	12-20 week males vs. 12-20 week virgins	5.32	<0.01
C ₅₇	30-40 week males vs. 30-40 week breeders	2.95	<0.01
C ₅₇	50-60 week breeders vs. 50-60 week virgins	2.61	<0.02
C ₅₇	All males vs. all virgins	3.34	<0.01
C ₅₇	All breeders vs. all virgins	3.20	<0.01
<i>Strain differences</i>			
C ₅₇ and A	All males vs. all males	3.25	<0.01
C ₅₇ and A	All breeders vs. all breeders	5.75	<0.01

Alkaline phosphatase levels in the three strains of mice average 4.6 units per 100 ml. serum for males, 4.4 units per 100 ml. serum for breeder females, and 6.3 units per 100 ml. serum for virgin females. These are relatively low values when compared with the average alkaline phosphatase activity of rat serum reported by Tuba, Baker, and Cantor (5) to be 113 units per 100 ml. for males, and 70 units per 100 ml. for breeder females. Nevertheless, variations in levels of the serum enzyme in mice are of sufficient magnitude to show that statistically significant effects of sex, previous pregnancy, and strain do exist. It appears that activity of the enzyme decreases in older males and virgin females of all strains. The average value for virgins is higher than for breeders or males of the same strain, but since it is difficult to obtain breeders in the youngest age group, it is not possible to say whether this difference is real. There are two statistically significant instances of strain differences. Greenstein (2) has tabulated the activity of various enzymes in normal tissues of mice of inbred, cancer-susceptible strains, including the C₅₇ and A strains. He has stated that no significant sex differences were observed with any of the tissues although a few strain differences were noted, e.g. catalase. However, the studies did not include serum enzymes. Zorzoli (8) has found that the acid and alkaline phosphatases in livers of C₅₇ mice are higher up to 20 days than at any subsequent age.

In general, the trends in Table I are similar to those that we found with serum tributyrinase in the C₃H, C₅₇, and A strains (4). Once more there is no indication of any correlation between enzyme levels and susceptibility or resistance to mammary tumors.

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ON THE ORIGIN OF HUMAN PLASMA PROTEINS:
ELECTROPHORETIC ANALYSES IN SELECTED
PATHOLOGICAL STATES¹

BY E. GORDON YOUNG AND R. V. WEBBER

Abstract

The pattern of human plasma proteins has been determined by electrophoretic analysis in 160 cases representing various pathological conditions. The concentration of albumin was lower than normal in 83% of the cases examined and the depression was most marked in cases of malnutrition and nephrosis. It is interpreted as indicative of the use of serum albumin as a reserve protein pool and of deterioration in the nutriture of persons who are ill. A rise in the level of fibrinogen was observed in 55% of the cases, frequently in the absence of infection. The constancy of α_1 -globulin between 0.25 and 0.50% was notable. The concentration was lowered in lymphatic leukemia and chronic hepatitis, and raised rarely. There were greater fluctuations in α_2 -globulin; a rise above 0.9% occurred in lymphatic diseases, nephrosis, and bone and joint conditions. A fall below 0.5% occurred only in hepatic portal cirrhosis. The level of β -globulin was relatively constant. It was raised most notably in nephrosis and disturbances of the bone marrow. Frequent fluctuations of the γ -globulins were observed, and a rise occurred most constantly in chronic hepatic infection and disturbances in the bone marrow but not in lymphatic diseases. Some evidence is presented showing the level of γ -globulin as inversely proportional to the number of lymphocytes. The importance of some unidentified cells of the bone marrow as a site of the origin of plasma proteins is stressed, with respect to α_2 -, β -, and γ -globulins.

Introduction

While there is good evidence for the origin of serum albumin, fibrinogen, and prothrombin of blood plasma from mammalian liver, there are comparatively few observations to suggest a possible origin of gamma-globulins from lymphatic cells. The origin of alpha- and beta-globulins is almost unknown. Several methods have been used in attempts to obtain evidence for the site of origin of the various plasma globulins and the subject has been adequately reviewed (18, 32, 52, 55). All methods are based on certain assumptions which make final conclusions difficult.

Since Tiselius developed his technique of electrophoresis in 1937 numerous analyses have been done on blood from many pathological conditions, initiated by Longsworth, Shedlovsky, and MacInnes (27) and directed mostly to diagnostic purposes. Of all pathological states the most intensively studied have been myelomatosis, nephritis, tuberculosis, and liver diseases. In other conditions published observations have been rather fragmentary.

By a selection of suitable cases, with involvement especially of lymphoid tissues, it was hoped to obtain more evidence for or against the origin of gamma globulins from this source. As the work progressed our interest was focused on diseases involving the bone marrow with their abnormal pattern of plasma proteins. Opportunity arose to examine blood from a number of other pathological conditions.

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Contribution from the Department of Biochemistry, Dalhousie University, Halifax, N.S., and the National Research Council of Canada, Maritime Regional Laboratory, Halifax, N.S.

Case histories were carefully read and the final diagnosis with supporting evidence was reviewed. Doubtful cases were not included in the following tables. The probability of erroneous diagnosis in a number of cases however must be considered. The investigation has extended over a period of three years.

Methods

Samples of oxalated blood sent to us from various hospitals were centrifuged within a few hours after withdrawal of the blood. The plasma was stored at 2° C. until analyzed. It was routinely diluted with barbiturate buffer in the ratio of 1 : 2.5 and dialyzed, with gentle mechanical stirring, in cellophane tubing against 1500 ml. of buffer solution for two hours or more at 20° C. The diluted plasma was poured into a small graduated cylinder and the dialyzer was rinsed twice with 1-ml. portions of the buffer solution. The rinsings were added to the plasma and the whole was then diluted to give a final ratio of 1 : 3. This ratio was occasionally altered if the total protein in the plasma was suspected to be unusually high or low. The composition of the buffer solution was 0.1 M sodium diethyl barbiturate, 0.02 M diethylbarbituric acid, and 0.1 M sodium chloride, to furnish a pH of 8.5 and an ionic strength of 0.22.

The concentration of total protein was determined by means of a dipping refractometer, assuming a specific refractive increment of 0.002. It should be understood that there is a source of error in any single method of estimating total proteins in plasma.

Electrophoresis was carried out for about 10 hr. in the Longsworth modification of the Tiselius apparatus at 1° C. with a current of 12 ma. and 70 v. Enlargements were made on graph paper and areas were measured with a planimeter in the usual manner (50). The absolute concentrations of the various components were then calculated from the figure for the total protein. In our opinion this is the only method of expression which permits of safe comparison and which eliminates distortion of distribution due to one or more abnormal components.

Normal Values

Limits of normal fluctuation have varied considerably in results previously published. It was necessary to arrive at a set of values for evaluation of normality and it seems useful to assemble the data, based on similar techniques, available at present. These are shown in Table I, including our own control series. Other values for average figures only, in the literature, have been omitted except those of Routh *et al.* (45) for children of three to eight years as they are the only absolute data available for children. The data for our own series of eight normal individuals are listed in Table II. They agree with previous observations and rather closely with those of Sterling (47) and Petermann and Hogness (39). The limits of fluctuation to be considered as normal were evaluated from the data in Table I. For general use investigators in this field must accept some such ranges and the limits stated represent those of over 200 adults of both sexes.

TABLE II
PATTERN OF PLASMA PROTEINS IN SERIES OF NORMAL PERSONS (GM. PER 100 ML. OF PLASMA OR SERUM)

Results

The possible relationships of lymphoid tissue and the reticulo-endothelial system with plasma proteins have been supported by some experiments and observations. For many years the origin of antibodies has been linked with lymphatic tissue and lymphocytes in particular (12, 13, 20). Antibodies are now definitely recognized as gamma globulins (13, 14, 22, 44, 50, 54) and this fraction tends to be elevated in some cases of chronic infection,—especially in the later stages. Destruction of lymphoid tissue gives rise to decreased production of antibodies; stimulation increases it (31, 36, 37). The investigation of the plasma proteins in cases exhibiting pathological activity of lymphoid tissue led to the following observations.

Lymphatic Diseases

The patterns of plasma proteins in some lymphatic diseases are shown in Table III which includes three cases of Hodgkin's disease (lymphogranulomatosis), three of generalized lymphadenitis, and one of lymphosarcoma. As a group they are remarkably similar showing definitely lowered levels of albumin, total protein generally within normal limits, and raised α -globulins and fibrinogen. This is particularly true of Hodgkin's disease. This result is in agreement with the only comparable observations of Petermann *et al.* (41). The rise in α -globulins has been attributed to the appearance of an acidic protein, probably a mucoprotein, not separable by electrophoresis at pH 8.6, and possibly associated with systemic intoxication (40).

The pattern is essentially the same for our three cases of lymphadenitis but not consistently so. In one case only, No. 59, was the gamma-globulin slightly raised.

Our single case of lymphosarcoma showed lowered albumin and slightly raised α_1 -globulin and fibrinogen. Petermann *et al.* (41) noted mainly a fall in albumin in their four cases and a rise in α -globulins and fibrinogen in one case.

Chronic Lymphatic Leukemia

Seven cases were examined as shown in Table IV. One of these, No. 44, was so unusual that it was not included in the averages. Despite the increases, sometimes enormous, in the numbers of lymphocytes, the plasma proteins are disturbed astonishingly little. A moderate decrease in total protein due to albumin is notable. This was essentially the finding of Petermann *et al.* (41) and Brown *et al.* (8). There was no evidence of raised alpha-globulin. Only two individuals in our series showed abnormally low levels of gamma-globulin and these exhibited the highest cell counts.

One patient with acute aleukemic leukemia exhibited lowered serum albumin and markedly raised gamma globulin with a concomitant leukopenia. Bieler, Ecker, and Spies (3) observed in addition a rise in α -globulin.

TABLE III
PATTERN OF PLASMA PROTEINS IN LYMPHATIC DISEASES (GM. PER 100 ML. OF PLASMA OR SERUM)

Case No.	Sex	Age	Total protein	Albumin	Globulins			Additional data
					α_1	α_2	β	
23	M	70	6.00	2.72	0.56	0.61	0.92	0.56
39	M	39	6.05	2.03	0.54	1.04	0.91	0.72
				7.52	2.67	0.56	1.27	0.91
88	M	63	7.00	2.15	0.61	1.11	1.09	1.02
147	M	23	6.10	2.20	0.54	1.02	0.67	1.14
59	M	14	7.16	2.15	0.57	1.10	0.79	0.88
99	F	37	4.52	2.16	0.21	0.56	0.72	Hodgkin's disease
148	M	15	5.16	2.53	0.31	0.70	0.34	Tuberculous lymphadenitis
Av.			6.19	2.33	0.49	1.06	0.85	0.94
							0.77	0.91
								Lymphosarcoma
								Hodgkin's disease, 3 mo. later
								Tuberculous lymphadenitis with leukopenia

TABLE IV
PATTERN OF PLASMA PROTEINS (GM. PER 100 ML. PLASMA OR SERUM)

TABLE V
PATTERN OF PLASMA PROTEINS (GM. PER 100 ML. OF PLASMA)

Sarcoidosis and Silicosis

Of four cases diagnosed as sarcoidosis, one showed hyperproteinemia and three definitely increased gamma-globulin. One was essentially normal as to plasma proteins. Considered as averages our results agree with those of Seibert *et al.* (46) and Fisher and Davis (16) indicating a high normal or raised level of total protein and hypergammaglobulinemia.

No previous observations appear to have been made in cases of silicosis. Case No. 115 presented a normal distribution but case No. 100 showed raised levels of α_2 - and β -globulins and fibrinogen. The results are listed in Table V.

Conditions with Increased Number of Plasma Cells

The origin of antibodies in plasma cells was proposed many years ago. Bing and Plum (5) and Bing (4) observed hyperglobulinemia in cases exhibiting an increase in plasma cells. While there appear to be exceptions to this relationship, Björneboe (6) has found that plasma cells in bone marrow are particularly significant. Fagraeus (15) came to the conclusion that the stimulation of the reticulo-endothelial system by antigens resulted in formation of plasma cells with concomitant production of antibodies, especially in the spleen.

This hypothesis led us to investigate cases exhibiting splenomegaly and those involving disturbances in the bone marrow.

Myelogenous Leukemia

Eight cases, classified as acute, subacute, and chronic, have been examined as shown in Table VI. Four of them exhibited comparatively high white cell counts. The pattern of plasma proteins was close to normal on the average, except for a slightly lowered level of albumin and raised fibrinogen. Two cases showed definitely raised gamma-globulin. As in lymphatic leukemia these changes are minor and insignificant as compared with the profound pathological changes involved. They agree with previous findings (8, 41).

Bone and Joint Diseases

As recorded in Table VII we have examined four cases of arthritis, three of osteomyelitis, and four of myelomatosis. In several ways they are the most interesting and revealing cases. All showed normal or *raised* total protein, *markedly* reduced albumin, and elevated globulins. Of the latter, α_1 and α_2 tended to be raised in arthritis and osteomyelitis but not in myelomatosis. Beta-globulin was higher than normal in most cases of osteomyelitis and very high in two of the four cases of myelomatosis. Fibrinogen was raised in most bloods of this group, frequently above 1%. Gamma-globulin was definitely higher than normal in rheumatoid arthritis, in three of the four cases of osteomyelitis but in only one case of myelomatosis. It would thus appear that in our four cases of multiple myeloma the Bence-Jones protein migrated in the β -component in two, in the γ -component in one and was absent in the fourth case.

TABLE VI
PATTERN OF PLASMA PROTEINS IN MYELOGENOUS LEUKEMIA (GM. PER 100 ML. OF PLASMA)

Case No.	Sex	Age	Total protein	Albumin	Globulins			γ	Additional data
					α_1	α_2	β	ϕ	
19	M	28	5.22	2.11	0.54	0.86	0.53	0.50	Acute, w.b.c. 153,600
24	F	36	7.43	4.26	0.25	0.81	0.85	0.37	Chronic, 5 yr., w.b.c. 8750
30	M	68	7.32	2.53	0.43	0.39	0.61	1.00	Chronic, 1 yr., w.b.c. 10,000, anemia and hyperplastic marrow
31	F	50	5.64	1.72	0.37	0.39	0.54	0.82	8 mo. later
35	M	73	6.38	3.02	0.32	0.49	1.00	0.55	Chronic, w.b.c. 23,000
69	M	31	6.05	2.64	0.36	0.55	0.52	0.71	Subacute, 2 yr., w.b.c. 112,000
96	F	56	7.68	4.57	0.27	0.69	0.69	0.41	Chronic, 6 mo., w.b.c. 30,000
142	M	32	6.30	3.58	0.38	0.54	1.41	0.65	Chronic, 6 mo., w.b.c. 201,000
				3.70	0.30	0.73	0.59	0.36	Chronic, w.b.c. 208,900
					3.13	0.36	0.61	0.77	1.07
					6.63				Av.

TABLE VII
PATTERN OF PLASMA PROTEINS IN BONE AND JOINT DISEASES (GM. PER 100 ML. OF PLASMA OR SERUM)

Case No.	Sex	Age	Total protein	Albumin	Globulins			Diagnosis
					α_1	α_2	β	
17	F	9	7.04	2.05	0.67	1.37	0.93	Rheumatoid arthritis
54	M	6.52	2.18	0.57	0.99	0.90	—	Rheumatoid arthritis, 1 yr.
161	F	8.06	2.34	0.52	0.83	0.95	0.94	Rheumatoid arthritis, 10 yr.
Av.			7.21	2.19	0.59	1.06	0.93	
118	M	59	5.72	2.83	0.31	0.70	1.13	Osteoarthritis
127	M	14	7.00	1.86	0.49	0.84	1.46	
131	F	?	10.40	1.79	1.11	1.59	1.52	
			7.60	1.40	0.71	1.03	1.23	
133	M	33	5.60	2.30	0.31	1.03	0.50	
Av.			7.70	2.32	0.64	1.15	1.16	
61	M	74	8.36	1.95	0.36	0.54	0.67	Myelomatosis, 4 yr.
90	M	63	6.84	3.25	0.34	0.73	1.33	Myelomatosis, several months
122	M	64*	10.04	1.40	0.36	0.58	0.98	—
149	M	56	8.60	2.18	0.26	0.51	0.72	Myelomatosis, 3 yr.
			9.52	2.26	0.51	0.63	0.42	Myelomatosis, 3 mo.
			9.80	2.21	0.22	0.45	0.68	Myelomatosis, 2 weeks later
Av.			8.46	2.20	0.33	0.59	3.43	Myelomatosis, 3 weeks later
112	F	46	4.96	2.50	0.29	0.41	0.93	Myelosclerosis

Several observations have been made in cases of rheumatoid arthritis indicating rather variable results. Our results agree with those of Dole *et al.* (11), Malmros and Blix (33), and Perlmann and Kaufman (38).

No previous observations appear to have been published on patients with osteomyelitis. Patterns observed differed from the normal very markedly and all globulins with the possible exception of α_1 were increased. The level of albumin was notably low. The pattern was very similar to that in advanced tuberculosis (46).

Our findings in myelomatosis fit the present conception that the abnormal constituent may occur in one of several places in the electrophoretic diagram (1, 8, 19, 23, 33, 35). No instance of Bence-Jones proteinuria occurred in our group of four cases.

One case of myelosclerosis with hypoplasia of the bone marrow was examined as shown in Table VII. It is an interesting pattern exhibiting hypoproteinemia with respect to albumin, and all globulins, excepting beta, were at the lower limits of normal fluctuation.

Liver Disease

We have examined 23 cases exhibiting various pathological aspects of liver disease. The present confusion in the classification of these conditions makes the analysis of the results difficult. In consequence averages only are presented in Table VIII, together with one notable case, grouped into primary infectious and chronic hepatitis, secondary obstructive jaundice, and some miscellaneous conditions. In this series it is apparent that the level of total protein was slightly below normal only in the condition of portal cirrhosis. Serum albumin was depressed in all patients except two. The condition of biliary cirrhosis was most marked in this respect with values mostly between 1 and 2%. Alpha₁-globulin was remarkably constant and within normal limits. Alpha₂-globulin was definitely lowered in the two cases of portal cirrhosis and also in a number of cases of acute infectious hepatitis. Beta-globulin was generally within normal limits with only an occasional high value. Gamma-globulin tended to be higher than normal in the four major groups. It was highest in chronic hepatitis and higher than normal in 85% of the cases. This was true for 75% of cases of acute hepatitis and about 50% of chronic hepatitis with portal cirrhosis and with obstructive jaundice. Fibrinogen also tended to be higher than normal in 50 to 100% of the four major groups.

The changing pattern in one patient (No. 81) with chronic hepatitis followed for over two years, is shown separately in Table VIII because of the notable features of very low albumin, very high globulin, and depressed α - and β -globulins.

The pattern in one patient with chronic hepatic insufficiency, associated with secondary anemia, leukopenia, and hyperplasia of the bone marrow, was abnormal in most components. This should probably be expected and it presents another case in which a disturbed bone marrow is associated with a

TABLE VIII
PATTERN OF PLASMA PROTEINS IN LIVER DISEASE (GM. PER 100 ML. OF PLASMA OR SERUM)

Diagnosis	No. of cases	Total protein	Albumin	Globulins				
				α_1	α_2	β	ϕ	γ
Acute infectious hepatitis	4	6.86	2.29	0.29	0.53	1.25	0.57	1.94
Chronic hepatitis, biliary cirrhosis	6	6.39	1.74	0.27	0.55	1.07	0.65	2.12
Chronic hepatitis, portal cirrhosis	2	5.65	2.18	0.22	0.33	0.83	0.57	1.54
Obstructive jaundice, due to various causes	7	6.67	2.81	0.40	0.80	1.20	0.60	0.69
Physiological hyperbilirubinemia	1	6.27	4.08	0.17	0.36	0.81	0.26	0.58
Chronic hepatic insufficiency, with anemia, leukopenia, and hyperplasia of bone marrow	1	9.44	2.87	0.73	0.98	1.49	—	3.38
Chronic hepatitis, Case No. 81								
2 weeks later		8.88	1.85	0.20	0.38	0.86	0.50	5.08
4 months later		7.08	1.44	0.20	0.31	0.77	0.48	3.87
2 years later		5.40	0.97	0.18	0.41	1.20	0.36	3.09
					0.24	0.54	0.36	3.10

markedly abnormal distribution of plasma proteins accompanied by hyperproteinemia, especially the gamma fraction. The analyses in the case of so-called physiological hyperbilirubinemia are taken as within normal limits.

Our results agree with the previous general findings of decreased albumin and increased γ -globulin with much less certain changes in other proteins (17, 21, 34, 43, 48, 51). These changes were most marked in chronic hepatitis.

Nephritis and Nephrosis

In renal disease the most marked changes in pattern are usually observed. This is particularly true of nephrosis in young children to a point when it becomes difficult to identify the components. In Table IX are presented the results from patients with nephrosis contrasted with those with nephritis. The profound nature of the disturbance in metabolism of plasma proteins in nephrosis is notable by effects on total protein, albumin, α_2 -, β - and γ -globulins, and fibrinogen, comparable with disturbances of the bone marrow. In all cases total protein and albumin were markedly lowered and α_2 -globulin raised. In about half of the cases β -globulin and fibrinogen were raised and γ -globulin lowered. Most of the patients were children of two to three years. In nephritis the notable features were lowered albumin and occasionally raised α_2 -, β -, and γ -globulins and fibrinogen. Our results in nephrosis are in accord with previous observations (26, 28, 45).

Blood Diseases

We have also examined blood samples from patients with various clotting abnormalities including thrombocytopenia, purpura, polycythemia, hemophilia, and other hemorrhagic diatheses. They showed generally normal total protein, moderately lowered albumin, and normal globulins with few exceptions. In one of two cases of thrombocytopenia purpura, followed for a period of six months, there were three components in the α -globulins, which corroborates a disturbance in this part of the plasma pattern as observed by Bernfeld *et al.* (2).

Malnutrition

The averages for six cases diagnosed as primary or secondary malnutrition are listed in Table IX. They show the characteristic pattern of lowered total protein and albumin. In all cases examined the level of fibrinogen was definitely raised. This was also essentially the picture in seven cases of carcinoma except that the total protein was usually within normal limits. Alpha₂-globulin was occasionally definitely raised. In one patient with carcinoma of the ovary the level of total protein reached 8.8% and γ -globulin 3.56%. Our major findings are in agreement with those of other investigators (7, 9, 39). A rise in the level of the α -globulins in cases of carcinoma has been observed and this occurred for α_2 in two of our seven cases.

TABLE IX
PATTERN OF PLASMA PROTEINS IN RENAL AND METABOLIC DISEASES (GM. PER 100 ML. OF PLASMA)

Diagnosis	No. of cases	Total protein	Albumin	Globulins			
				α_1	α_2	β	ϕ
Nephrosis	7	4.32	0.55	0.52	1.63	1.20	0.79
Nephritis	9	6.32	2.38	0.47	0.81	0.91	0.59
Malnutrition	6	5.43	2.09	0.45	0.75	0.81	0.70
Carcinoma of various organs	7	6.04	2.11	0.46	0.86	0.75	0.65
Endocrine diseases							
Diabetes mellitus	5	5.97	2.17	0.36	0.56	0.89	0.79
Hypothyroidism	3	6.58	3.19	0.42	0.58	0.84	0.53
Hyperthyroidism	1	6.76	2.98	0.37	0.39	1.46	0.55
Acromegaly	1	5.72	3.38	0.18	0.44	0.94	0.31
Hypopituitarism	1	6.40	3.01	0.29	0.13	1.25	0.48
Hyperparathyroidism	1	6.08	2.31	0.60	0.84	0.75	0.84
Addison's disease	3	6.80	3.48	0.28	0.73	0.90	0.48

Endocrine Diseases

The pattern of plasma proteins in endocrine diseases has received relatively little attention except for the observations of Lewis and his co-workers (24, 25). Fifteen analyses representing seven pathological states are shown in Table IX. The usual reduction in the level of albumin is apparent. The only other abnormality is the rise in fibrinogen in four of the five patients with diabetes mellitus. Lewis *et al.* (25) did not observe this but noted an occasional high β -globulin.

In one case of hypothyroidism there was a moderate increase in β -globulin as previously reported by Lewis and McCullough (24).

In one case of hypopituitarism with tumor of the gland there was the very unusual occurrence of a reduction in α_2 -globulin.

Injections of adrenocorticotropic hormone has been shown to induce lymphopenia (53) and a simultaneous rise in β - and γ -globulins (54). This suggested to us the investigation of the plasma proteins in cases of Addison's disease to determine whether decreased activity of the adrenal would show any concomitant effects on the pattern of plasma proteins. The averages of three cases are presented in Table IX and are within normal limits. All patients were showing a slight lymphocytosis at the time of examination. Luetscher (29) found only a fall in the level of albumin in this condition.

Discussion

By a selective consideration of the cases examined and by the elimination of all repetitions, the frequency of abnormal values in the levels of the plasma proteins may be calculated from our results comprising 127 individuals as shown in Table X. The limits of normal variation adopted are stated above.

The most frequent abnormality was a lowered level of serum albumin which occurred in no less than 83% of the cases. This is confirmatory of many previous observations summarized by Luetscher (30) in the words, "The common denominator of almost every pathological state is a relative or absolute decrease in serum albumin". Malnutrition, hepatic dysfunction, albuminuria, and some other factors may play a part in this condition. It

TABLE X
FREQUENCY OF VARIATION FROM NORMAL LIMITS

	Raised, %	Lowered, %
Total protein	8	33
Albumin	0	83
α_1 -Globulin	5	9
α_2 -Globulin	17	15
β -Globulin	15	3
Fibrinogen	55	0
γ -Globulin	29	6

obviously represents a very common condition and emphasizes the need for closer study of hepatic function in many pathological states. It suggests the possibility of decreased powers of synthesis of albumin, or alternately a remarkably common deterioration in the nutriture of persons who are ill. Losses of albumin by way of the kidney or by exudation occurred in a relatively small number of our cases.

The frequency of raised levels of fibrinogen holds second place and no less than 55% of all our cases exhibited this abnormality. The conditions in which half or more of our cases showed this increase were malnutrition, inclusive of malignancy, lymphoid and myeloid diseases, hepatic and renal diseases, chronic infections such as tuberculosis and arthritis, and diabetes. The frequency of increased levels does not appear to have been emphasized previously. Chronic infection as the etiological agent is not sufficient to explain observed alteration. Moderate hepatic dysfunction deserves further investigation in this regard, and it may be that abnormal levels of fibrinogen indicate the most sensitive index of liver dysfunction. The failure of the ϕ peak to separate completely from the β peak may be the correct explanation.

The relative constancy of α_1 -globulin is a notable feature of this series. The level was raised in osteomyelitis, Hodgkin's disease, rheumatoid arthritis, and occasionally in renal disease. This suggests a possible specific link with bone tissue and does not relate this globulin with the liver. The level was lowered in lymphatic leukemia and chronic hepatitis.

Alpha₂-globulin was raised in lymphatic diseases, nephrosis, and bone and joint conditions. It was lowered only in portal cirrhosis. While not indicative of any special tissue, emphasis should be placed on the relationship with bone tissue as in the case of α_1 -globulin.

Beta-globulin was remarkably constant and within normal limits in most conditions. It was raised most notably in nephrosis, disturbances of the bone marrow, and occasionally in chronic hepatitis. The proposed relationship with lipid metabolism and hyperlipaemia would fit our observations. Again, however, there is a link to disturbances in the bone marrow.

Gamma-globulin was raised frequently, especially in liver diseases of all types, in tuberculous osteomyelitis, in arthritis, in sarcoidosis, and occasionally in other diseases. It was lowered in nephrosis. It was not abnormal in 15 of our 16 cases of leukemia, lymphatic and myelogenous, but it was definitely raised in the only case of acute aleukemic leukemia observed. It was not raised characteristically in moderate to advanced tuberculosis, but it was in sarcoidosis. Chronic infection cannot explain these results on the basis of hyperimmunity. The role of the liver is not clear although this organ obviously plays a part. Very high counts of lymphocytes may be related to a lowered level of γ -globulin. The condition of leukopenia was frequently accompanied by a rise in level.

One case of acromegaly is interesting in that the level of total protein, α_1 -, and α_2 -, and γ -globulins, were all below normal.

The major finding in this series of analysis is the importance of the bone marrow, or at least bone and joint diseases, in relationship to the pattern of plasma proteins. This is not surprising if consideration is given to the amount of this tissue present in the human body, and its activity as the site of origin of the formed elements of the blood. The theory of Whipple of the close interrelationship of all proteins of the blood and the conversion of plasma proteins to hemoglobin is definitely compatible with the finding of the importance of marrow cells in the metabolism of plasma proteins, especially α_2 -, β -, and γ -globulins. The relationship of marrow cells to plasma cells and of plasma cells to the reticulo-endothelial system must be left to the histologist to unravel.

The lack of specificity of the schlieren diagrams of plasma proteins in relation to practically all diseases is now recognized. This is confirmed by our observations. Stern and Reiner (49) have stated that "The protein spectrum in plasma and serum is the resultant of a host of factors concerned with the formation, the interaction, and the destruction of the individual components". This is speculation. Luetscher (30) has stated it in similar terms, "The picture has proven to be characteristic not of specific disease but of the host's reaction to infection or injury. The various changes are frequently proportional to the severity of the physiological disturbance." There is need for a closer liaison between histology and biochemistry.

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CULTIVATION OF POLIOMYELITIS VIRUS IN TISSUE CULTURE

IV. FURTHER OBSERVATIONS ON VIRUS PROPAGATION IN HUMAN TISSUES WITH A SYNTHETIC NUTRIENT MEDIUM¹

BY A. E. FRANKLIN, D. DUNCAN, W. WOOD, AND A. J. RHODES

Abstract

This paper presents further observations on the propagation of the Lansing strain of poliomyelitis virus in Maitland-type cultures of human tissue derived from embryonic brain and cord and from kidney, in a synthetic nutrient medium. The survival time of cultures of human embryonic brain and cord was previously found to be over 70 days, and we now report that cultures of human embryonic kidney have survived for over 100 days. Virus has been detected in the supernatant fluids of cultures of brain and cord for more than 60 days, and of kidney for more than 100 days. Virus titers of more than $10^{-2.0}$ have been obtained in cultures of human embryonic kidney. Human tonsillar tissue has survived for more than 50 days in cultures, and virus has been detected in the supernatant fluids for more than 40 days. Studies on glucose utilization have been of value in estimating the level of metabolism of these tissues.

Introduction

We have recently reported (2, 11, 14) that the Lansing poliomyelitis virus can be grown in cultures of tissues derived from human embryonic organs and monkey testis, supplied with nutrient in the form of Hanks-Simms' medium or a synthetic mixture, No. 199, devised by Morgan, Morton, and Parker (4, 6). A striking feature of the cultures in which the synthetic mixture was employed was the prolonged survival of the tissues; human embryonic brain and cord, for example, surviving for more than 70 days. Glucose utilization and pH changes in the supernatant fluids of the cultures were estimated routinely. Associated with the prolonged survival of tissue was the liberation of the virus into the supernatant fluid over a lengthy period; virus was detected in the fluids of cultures of human embryonic brain and cord for more than 60 days.

The present paper confirms previous results with human embryonic brain and cord cultures. We now report that human embryonic kidney and human postnatal tonsillar tissue can also be used satisfactorily as the source of tissue for the propagation of the Lansing strain. Both kidney and tonsil have been studied by other workers, but not in cultures with a synthetic nutrient. Thus, Robbins *et al.* (8) used normal kidney tissue obtained at operation from infants as the source of tissue in cultures employed for virus isolation and serological identification. Smith, Chambers, and Evans (9) reported on the virus growth supporting properties of tonsillar tissues from children three to five years old. These tissues did not, however, prove to be satisfactory, as in only a few of the several cultures prepared was there any evidence of virus multiplication.

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Methods

The modified Maitland technique that was used followed fairly closely the procedures employed by Enders and his associates in the cultivation of mumps virus (12) and poliomyelitis virus (1, 8, 13) in human tissues. Human embryos were obtained from the gynaecological services of the Toronto General Hospital and Women's College Hospital, Toronto. Embryo No. 13 was 9 in. long, and the heart was beating slowly and irregularly at the time of arrival in the laboratory; considerable haemorrhage was observed throughout the brain. Embryo No. 15 was 11 in. long, and the heart was beating strongly. Brain, cord, and kidneys were removed from both of these embryos. Tonsils were obtained from the Tonsil Suite, Hospital for Sick Children, Toronto, and were placed immediately in mixture No. 199 containing 200 units of penicillin and 200 μ gm. streptomycin per ml.

Selected tissues were washed two or three times in a bath of mixture No. 199 containing 100 units of penicillin and 100 μ gm. of streptomycin per ml. The tissues were then suspended in a small amount of this solution and chopped finely with scissors to form fragments about 1 mm. in diameter. The minced tissue was washed several times in nutrient, and the excess fluid removed. Four drops of the concentrated suspension were then pipetted into each of six 25-ml. Erlenmeyer flasks to which had been added 3.0 ml. of Mixture 199. The flasks were tightly stoppered and incubated at 37°C.

The next day, the fluid elements of the cultures were removed and replaced with 3.0 ml. of fresh nutrient containing half the above-mentioned quantities of antibiotics. Four flasks were then infected with 0.1 ml. of virus-containing material, either a 10% suspension of infected mouse central nervous system, or undiluted fluid from a previous culture. The remaining two flasks served as controls, and were not inoculated with virus.

The supernatant fluids of the cultures were changed two days after addition of virus; subsequent changes were made every three or four days. At the time of making fluid changes, the supernatants were removed as completely as possible, leaving not more than 0.2 ml. of residual fluid. Three milliliters of Mixture 199 containing 50 units of penicillin and 50 μ gm. of streptomycin per ml. were then added. Fluids from the virus-infected flasks were pooled, and after centrifugation at 2000 r.p.m. for 10 min. the supernatants were stored in a dry-ice box. Fluid changes were carried out in the uninfected flasks in a similar manner.

The values given for glucose utilization are based on estimations carried out on pooled fluids from four infected cultures and also on pooled fluids from two uninfected control cultures unless it has been stated otherwise. The incidence of contamination has been observed to be extremely low, and, during the past year, there have been no cases of bacterial contamination and considerably less than 1% of cultures contaminated by yeasts and molds.

The amounts of glucose utilized by the tissues were estimated according to Somogyi's modification (10) of Nelson's technique (5). Tests for glucose were performed on the pooled fluids of the uninfected cultures, and also on

the pooled fluids of the infected flasks. The results are expressed in terms of the average percentage of glucose used per day. This figure is determined by estimating the percentage of the initial glucose content used during the period of three to four days of incubation, and dividing this figure by the number of days of incubation.

The least number of fluid changes made in any culture in the present series was 11, whereas the greatest number was 35. When it appeared from the results of tests for glucose utilization that the tissues in the infected flasks were no longer metabolizing, the cultures were terminated.

Subcultivation of virus was carried out by preparing new cultures of the same type of tissue, and inoculating them with 0.1 ml. of a suitable fluid change of the previous culture. Usually four flasks were inoculated with such virus-containing fluids, and two flasks with 0.1 ml. of fluids removed from the uninjected flasks at a corresponding time, to serve as controls. Flasks were then incubated and fluid changes made as above described.

Infectivity tests were carried out in male Swiss mice, 12-14 gm., supplied by the Connaught Medical Research Laboratories. The animals were inoculated intracerebrally with 0.03 ml. of culture fluids. In addition, the cells remaining at the conclusion of the culture were ground and the suspension inoculated into mice. These were examined daily for three weeks, but deaths occurring on the day following inoculation were not regarded as caused by virus. Titrations of virus content were carried out by inoculation of groups of 8-10 mice with serial dilutions of culture fluid. LD₅₀ endpoints were calculated by the Kärber method (3, 7). Several culture fluids were inoculated thalamically in rhesus monkeys (0.8 ml.). All animals were sacrificed for histological examination after four weeks or at the onset of paralysis. On several occasions, pooled fluids from the uninoculated flasks of a culture were inoculated into mice. Only very occasionally did such mice die, and these animals were not paralyzed. This procedure served to ensure that no mouse pathogenic agent derived from human tissue was being propagated unknowingly.

Neutralization tests with Lansing immune serum prepared in monkeys were carried out on fluids from several cultures. Mixtures of serum and infected fluids were allowed to stand for one hour at room temperature and were then inoculated in groups of 20 mice, along with suitable controls, to test for neutralization. In all cases, the infectivity of the culture fluids was specifically neutralized by Lansing antiserum.

Results

(A) *Propagation of Lansing Virus in Tissues from Human Embryonic Brain and Cord*

We have previously reported (11) two experiments in which cultures of tissue derived from human embryonic brain and cord were infected with Lansing virus (mouse central nervous system), and we now report a third experiment. In this experiment the cultures were found to be still using

glucose when the experiment was terminated after 64 days' incubation, and deaths were observed in mice inoculated with culture fluids collected over this entire period. The culture fluids of this series were not titrated in mice. However, the fluids of one of the earlier cultures (2) have now been titrated and the results are shown in Table I.

TABLE I
TITERS OF LANSING VIRUS PROPAGATED IN CULTURES OF HUMAN
EMBRYONIC BRAIN AND CORD WITH SYNTHETIC MIXTURE NO. 199*

Pooled culture fluids	Incubation (days)	LD ₅₀ titers determined in mice†
1	3	1.87
2	7	1.62
3	11	1.75
4	15	1.50
5	18	1.08
6	22	0.63
7	26	0.12
8	30	0.37
9	34	0.37
11	43	0.37
13	51	0.50
15	60	1.25
16	65	0.12
18	74	Nil

* Cultures inoculated with 500 LD₅₀ (for mice) of Lansing virus mouse pool.

† Negative logarithms of titers.

We have previously reported that Lansing virus has been carried through a primary culture and four successive subcultures (11). The nutrient medium in this series of experiments was Hanks-Simms' mixture. In the present series, we have continued subcultivation of the virus through the fifth and sixth subcultures, Mixture 199 being the source of nutrient.

The fifth and sixth subcultures of the virus in cultures of human embryonic brain and cord tissues were carried for 40 and 60 days respectively, and virus was produced throughout, as shown in Tables II and III. These periods do not necessarily represent the maximum period of survival of the tissues under these conditions. Studies on glucose utilization confirmed previous observations according to which the amount of glucose used was lower in infected cultures than in control, uninfected cultures.

A seventh subculture of the virus has been initiated with inoculum from the fourth fluid change of the sixth subculture. It can now be calculated that the dilution of the inoculum of mouse brain suspension added to the primary culture of the series is 10^{-45.7}, so that there is the strongest evidence that the Lansing virus has actually multiplied throughout the series of subcultures. It is evident, therefore, that Mixture 199 can be used for the routine subcultivation of Lansing virus in tissue cultures.

TABLE II

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF HUMAN EMBRYONIC BRAIN AND CORD (No. 13) WITH SYNTHETIC MIXTURE NO. 199: FIFTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	3	—	—	3/6
2	7	—	—	6/6
3	11	—	—	5/6
4	15	23.6	10.2	6/6
5	19	—	—	5/6
6	22	20.1	4.6	6/6†
7	26	12.9	3.7	6/6
8	29	10.9	3.0	3/6
9	33	7.5	3.1	2/6
10	36	5.5	2.1	4/6
11	40	4.2	2.7	3/6
Ground cells	40			6/6

* Cultures inoculated with 0.1 ml. of the fifth culture fluid from the fourth subculture of the virus in human embryonic brain and cord (No. 12) with a titer of approximately 20 LD₅₀ for mice.

† Positive infectivity test in monkey.

TABLE III

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF HUMAN EMBRYONIC BRAIN AND CORD (No. 15), WITH SYNTHETIC MIXTURE NO. 199: SIXTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	3	42.5	42.6	1/6
2	5	28.4	32.5	5/6
3	9	24.6	24.8	5/6
4	12	28.9	27.4	5/8†
5	16	15.6	13.3	3/8
6	21	9.3	3.7	3/6
7	24	8.6	5.3	0/6
8	28	7.3	3.7	0/6
9	32	6.4	3.2	1/6
10	36	5.4	1.4	2/6
11	40	4.3	1.4	0/6
12	44	3.9	0.7	2/6
13	47	2.0	1.8	1/6
14	50	4.7	1.5	0/6
15	53	13.5	0.9	2/6
16	57	0	0	3/6
17	60	11.6	6.9	0/6
Ground cells	60			3/5

* Cultures inoculated with 0.1 ml. of the sixth culture fluid from the fifth subculture of the virus in human embryonic brain and cord (No. 13) with a titer of approximately 100 LD₅₀ for mice.

† Positive infectivity test in monkey.

(B) *Propagation of Lansing Virus in Cultures of Human Embryonic Kidney*

We have previously carried Lansing virus through a primary culture and two subcultures (11) in cultures of human embryonic kidney in which Hanks-Simms' fluid served as nutrient. This series has now been continued through the third and fourth subculture with Mixture 199 as nutrient.

The results of tests for glucose utilization and mouse infectivity are summarized in Tables IV and V. It will be seen that the tissues survived for over 100 days in each experiment, and that virus was present in the culture fluids for the same period.

TABLE IV

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF HUMAN EMBRYONIC KIDNEY (No. 13) WITH SYNTHETIC MIXTURE NO. 199: THIRD SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	4	—	—	0/6
2	8	—	—	0/6
3	12	—	—	6/6
4	16	16.3	17.2	6/6
5	20	16.2	16.3	6/6
6	23	—	—	6/6
7	27	17.6	13.8	5/6†
8	30	17.8	13.4	6/6
9	34	19.1	12.3	8/8
10	37	18.8	12.4	6/6
11	41	14.2	13.1	6/8
12	44	12.4	12.1	6/6
13	48	10.3	8.3	7/8
14	51	8.6	10.1	5/6
15	55	6.2	6.7	8/8
16	58	3.2	5.2	6/6
17	62	7.5	4.5	8/8
18	65	7.0	5.4	6/6
19	69	3.2	5.1	8/8
20	72	3.6	4.4	6/6
21	76	0	0	8/8
22	79	4.7	4.9	6/6
23	83	4.7	5.1	6/6
24	86	2.4	4.6	6/6
25	89	5.4	0	6/6†
26	93	3.9	1.1	5/6
27	97	1.1	0	5/6
28	102	0.8	1.0	6/6
29	106	0.9	0.7	6/6
30	110	3.3	2.4	4/6
31	114	0.4	0	3/6
32	118	3.4	0.7	0/6
33	121	1.3	1.9	1/6
34	125	4.0	0.7	0/6
35	128	7.5	3.3	0/6
Ground cells	128			0/6†

* Cultures inoculated with 0.1 ml. of the fifth culture fluid from the second subculture of the virus in human embryonic kidney (embryo No. 6), with a titer of approximately 100 LD₅₀ for mice.

† Positive infectivity test in monkey.

TABLE V

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF HUMAN EMBRYONIC KIDNEY (No. 13) WITH SYNTHETIC MIXTURE NO. 199: FOURTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	3	47.6	46.0	5/6
2	5	50.0	50.0	5/5
3	9	25.0	25.0	6/8†
4	12	32.6	33.3	8/8†**
5	16	23.9	24.1	8/8†
6	21	18.7	18.4	7/8†
7	24	33.3	33.3	8/8†
8	28	23.4	24.0	7/8
9	32	23.0	14.8	6/8
10	36	22.7	22.7	5/6
11	40	15.8	24.1	4/6
12	44	18.8	22.2	5/6
13	47	30.8	20.0	4/6
14	50	33.3	18.1	6/6
15	53	32.8	12.1	3/6
16	57	0	9.4	6/6
17	60	3.9	21.3	6/6
18	64	16.1	2.9	6/6
19	67	7.8	1.6	4/6
20	71	1.8	2.9	6/6
21	74	3.5	2.6	3/6
22	78	0	0.8	6/6
23	81	0	0	6/6
24	84	1.9	0	4/6
25	88	1.8	0	1/6
26	92	2.7	1.3	5/6**
27	95	4.7	2.2	2/6
28	99	0.5	0.2	1/6
29	103	0.2	0	1/6
Ground cells	103			0/6**

* Cultures inoculated with 0.1 ml. of the seventh culture fluid from the third subculture of the virus in human embryonic kidney (embryo No. 13), with a titer of approximately 800 LD₅₀ for mice.

† Mice inoculated with a dilution of 1:10 of the culture fluid.

** Positive infectivity test in monkey.

Several of the culture fluids in these experiments were titrated by inoculation of mice (Tables VI and VII). These values represent the highest titers so far obtained in our laboratory.

A fifth subculture of the virus has recently been prepared in cultures of tissue derived from human embryonic kidney. The dilution of the virus inoculum originally added to this series of cultures in kidney tissue was 10^{-59.1}.

(C) Propagation of Lansing Virus in Cultures of Human Tonsils

Propagation of Lansing virus has been demonstrated in human tonsillar tissue in two separate experiments and Tables VIII and IX summarize the results of glucose utilization and mouse infectivity tests in the two series.

TABLE VI

TITERS OF LANSING VIRUS PROPAGATED IN HUMAN EMBRYONIC KIDNEY (NO. 13)
WITH SYNTHETIC MIXTURE NO. 199*

Pooled culture fluids	Incubation (days)	LD ₅₀ titers determined in mice†
3	12	3.23
4	16	3.13
5	20	2.63
6	23	2.88
7	27	2.38
8	30	2.38
9	34	3.13
11	41	1.63
13	48	2.00
15	55	2.23
17	62	1.50
19	69	1.50
21	76	1.63

* Cultures inoculated with 0.1 ml. of the fifth culture fluid from the second subculture of the virus in human embryonic kidney (embryo No. 6), with a titer of approximately 100 LD₅₀ for mice.

† Negative logarithms of titers.

TABLE VII

TITERS OF LANSING VIRUS PROPAGATED IN HUMAN EMBRYONIC KIDNEY (NO. 15) WITH SYNTHETIC MIXTURE NO. 199*

Culture fluid	Incubation (days)	LD ₅₀ titers determined in mice†
3	9	2.50
4	12	2.37
5	16	2.12
6	21	1.74
7	24	2.00
8	28	0.62
9	32	0.87
11	40	0.0
13	47	1.62
15	53	0.62
17	60	1.37
19	67	1.00
21	74	0.25
23	81	0.37
24	84	0.50
26	92	0.37

* Cultures inoculated with 0.1 ml. of the seventh culture fluid from the third subculture of the virus in human embryonic kidney (embryo No. 13), with a titer of approximately 800 LD₅₀.

† Negative logarithms of titers.

TABLE VIII

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF HUMAN TONSILLAR TISSUE (No. 1) WITH SYNTHETIC MIXTURE NO. 199: FOURTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	3	43.6	47.0	6/6
2	7	25.0	25.0	6/6
3	10	31.6	31.3	4/6†
4	13	28.5	27.8	6/6†
5	17	20.9	23.3	6/6†**
6	21	13.9	18.7	4/6
7	26	8.2	12.9	6/6
8	30	2.5	9.5	4/6
9	34	2.5	7.5	6/6
10	38	2.1	7.4	5/6
11	42	0	2.9	6/6
12	45	0	4.2	6/6
13	49	0.5	1.6	6/6
14	52	2.2	2.4	6/6†
15	55	0	0	3/6
16	58	0	0	3/6
Ground cells	58			4/6

* Cultures inoculated with 0.1 ml. of the fifth culture fluid from the third subculture of the virus in human embryonic kidney (No. 13) with a titer of approximately 1400 LD₅₀ for mice.† Titration values in mice for pooled culture fluids 3, 4, 5, and 14 were 10^{-1.75}, 10^{-3.00}, 10^{-2.55}, and 10^{-0.62}, respectively.

** Positive infectivity test in monkey.

TABLE IX

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF HUMAN TONSILLAR TISSUE (No. 2) WITH SYNTHETIC MIXTURE NO. 199: FIFTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	3	50.0	44.5	4/6
2	7	14.6	11.6	4/6
3	10	15.0	14.6	5/6†
4	14	9.7	11.7	6/6†
5	17	10.5	20.3	6/6†**
6	21	10.3	18.9	6/6
7	24	7.0	16.1	2/6
8	28	4.3	16.0	6/6
9	31	0	9.1	5/6
10	34	0	4.9	6/6
11	38	0	3.8	5/6†
12	42	0	2.2	6/6**
13	45	4.5	2.7	1/6
14	49	1.1	0.3	0/6
15	53	1.3	2.6	0/6
16	56	0.5	0.3	0/6
17	59	0.5	1.0	0/6
Ground cells	59			0/6

* Cultures inoculated with 0.1 ml. of the fifth culture fluid from the fourth subculture of the virus in human tonsillar tissue (No. 1) with a titer of approximately 1200 LD₅₀ for mice.† Titration values in mice for pooled culture fluids 3, 4, 5, and 11 were 10^{-2.00}, 10^{-1.82}, 10^{-1.0}, and 10^{-1.25}, respectively.

** Positive infectivity test in monkey.

It will be observed that more glucose was used in some of the cultures infected with the virus than in control uninfected cultures. This is in contrast to the inhibitory effect of the virus on glucose utilization observed in cultures of tissue derived from human embryonic brain and cord, and the explanation is not immediately apparent.

Discussion

The results recorded in this paper indicate that the Lansing poliomyelitis virus propagates in cultures of tissue derived from human embryonic brain and cord, human embryonic kidney, and human tonsils when a synthetic medium is used as nutrient. We have now shown that mixture No. 199 can be used successfully to replace Hanks-Simms' medium in several aspects of the study of poliomyelitis virus in tissue culture. It would appear that Mixture 199 allows a prolonged survival period of the tissue fragments, and a liberation of virus into the supernatant fluids for a long period. Furthermore, changes of culture fluids can be carried out at regular intervals, a procedure not possible when Hanks-Simms' medium is used. We have routinely changed the culture fluids twice weekly, a three-day interval being followed by one of four days. The titers of virus propagated in cultures supplied with Mixture 199 are as high as or higher than those found by ourselves and other workers in cultures in which Hanks-Simms' mixture was used.

A striking finding of the present work was the prolonged survival of tissue fragments from human embryonic kidney, and the demonstration of virus in the supernatant fluids for more than 100 days. Virus titers were also found to be higher than corresponding titers in cultures of human embryonic brain and cord.

It is also of considerable interest to note that virus could be propagated in cultures of tissue derived from human tonsils. In these experiments it was observed that the cultures did not utilize glucose for much more than 50 days, which is less than has been found with cultures of human embryonic tissues.

One of the main applications of our work lies in the production of large quantities of poliomyelitis virus for use in a vaccine, for skin testing, or for study by physicochemical methods. It appears that the stage has now been reached where experiments can be initiated to determine whether poliomyelitis virus will grow in containers considerably larger than those previously used. Evidently, human embryonic kidney, as well as human tonsil, may be considered as potential sources of tissue for such cultures. In work of this sort, synthetic Mixture 199 has a number of advantages over mixtures that contain animal organ extracts and serum. For example, the fact that the medium is of chemically-defined composition means that there are fewer variations in the constituents than when animal sera or embryo extracts are employed. Furthermore, the synthetic medium does not contain antigens of animal origin. It is of advantage that cultures metabolize for a lengthy

period when a synthetic nutrient medium is used, and that there is correspondingly a prolonged liberation of virus into the culture fluids. Thus, Lansing virus has been demonstrated in culture fluids after as long as 100 days.

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CULTIVATION OF POLIOMYELITIS VIRUS IN TISSUE CULTURE

V. OBSERVATIONS ON VIRUS PROPAGATION IN CERTAIN ANIMAL TISSUES WITH A SYNTHETIC NUTRIENT MEDIUM¹

BY D. DUNCAN, A. E. FRANKLIN, W. WOOD, AND A. J. RHODES

Abstract

Further observations have been made on the propagation of Lansing poliomyelitis virus in tissue cultures. It has been observed that tissues derived from several organs of rhesus monkeys will support virus growth in tissue cultures in Erlenmeyer flasks with a synthetic medium as the source of nutrient. Cultures of tissues from monkey testis, lung, kidney, and gut have survived for long periods, and virus has been regularly recovered even from fluids removed from cultures after as late as 125 days. Cultures of tissues from monkey brain and cord, and muscle, did not survive as long, and less virus was demonstrated in the supernatant fluids. Muscle from the diaphragm did not appear to support growth. Cultures of tissues from the brain, kidney, and lung of beef embryos survived for long periods, but no virus was found in any of the culture fluids.

Introduction

In preceding papers (1, 2, 9) we have reported that the Lansing poliomyelitis virus can be propagated in cultures of tissue derived from the brain and cord, and kidney, of human embryos. Tonsils from children have also proved satisfactory sources of tissue. In these experiments use was made of a synthetic nutrient, mixture No. 199 (3, 5). One of the objects of this work is the preparation of poliomyelitis virus in large amounts. For this purpose, it would be desirable to have a source of tissue more readily available than human embryos, and there would be many advantages in using material obtainable from the abattoir or from monkeys. In this connection, it should be noted that Smith, Chambers, and Evans (6) and Syverton, Scherer, and Butorac (8) demonstrated proliferation of the Yale-SK and Lansing viruses in flask cultures of testicular tissue from rhesus and cynomolgus monkeys, and we have confirmed these observations (9). It is the object of this paper to report that Lansing poliomyelitis virus can be grown successfully in flask cultures of tissue obtained from several organs of rhesus monkeys, but not in similar cultures prepared from the organs of beef embryos.

Methods

The general manner of preparation and maintenance of monkey and beef embryonic tissues in cultures in 25-ml. Erlenmeyer flasks was exactly as described in the preceding paper (2). Testes were removed from rhesus monkeys housed in the Connaught Medical Research Laboratories, University of Toronto; brain and cord, kidney, lung, gut, pectoral muscle, and diaphragm were also obtained. Beef embryos, about 18 in. long, were obtained from the abattoir of Swift Canadian Co. Ltd., Toronto. Mixture No. 199 (3, 5) was

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used in all these experiments as the source of nutrient. Tests for the glucose content of culture fluids were carried out according to Somogyi's modification (7) of Nelson's technique (4). A diagram representing the history of subcultivation of Lansing virus in tissue culture in our laboratory as described in this and previous papers is given on p. 82.

(A) *Propagation of Lansing Virus in Cultures of Tissue from Monkey Testis*

A preliminary experiment was carried out in which minced testis (monkey No. 11) was the source of tissue. These cultures were infected with virus and carried for 53 days. The results are summarized in Table I from which it will be observed that glucose utilization was very high even when the experiment was terminated. Virus was demonstrated in the supernatant fluids over this entire period.

In a confirmatory experiment (Table II), tissue was obtained from the testis of monkey No. 12. It will be noted that the tissues were still using glucose after 100 days of incubation, and that there was liberation of virus throughout the period. The results of a third experiment (Tables III and IV) are similar.

(B) *Propagation of Lansing Virus in Cultures of Tissues from Other Monkey Organs*

Cultures of the following monkey organs were infected with Lansing virus: brain, kidney, lung, gut, muscle, and diaphragm. The results are shown in Table III, from which it will be noted that the average per cent

TABLE I
GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF MONKEY TESTIS (No. 11) WITH
SYNTHETIC MIXTURE NO. 199: FOURTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid
		Uninfected	Infected	
1	3	48.2	48.2	3/5
2	5	50.0	50.0	1/6
3	8	27.7	28.9	4/6
4	12	23.7	23.9	6/6†
5	16	24.8	25.0	6/6
6	21	18.2	17.3	0/6
7	25	25.0	25.0	6/6
8	29	24.1	24.0	5/5
9	33	25.0	25.0	5/6
10	37	23.1	23.6	4/6
11	40	32.7	31.0	3/6
12	44	23.2	23.2	4/6
13	47	32.3	31.0	5/6
14	50	31.0	30.2	6/6
15	53	29.5	29.5	5/6
Ground cells	53			4/6

* Cultures inoculated with 0.1 ml. of the seventh culture fluid from the third subculture of the virus in human embryonic kidney (No. 13) with a titer of approximately 800 LD₅₀ for mice.

† Positive infectivity test in monkey.

glucose used per day in the cultures varied considerably from one tissue to another. The length of time in which glucose was utilized by the cultures also varied, but cultures of kidney, lung, gut, and testis showed glucose utilization for long periods. Infectivity tests in mice are summarized in Table IV, and it will be noted that the greatest evidence of virus propagation was found in cultures of testis, lung, kidney, and gut. The diaphragm was the only tissue that did not support virus propagation.

TABLE II

GLUCOSE UTILIZATION AND INFECTIVITY TESTS IN CULTURES OF MONKEY TESTIS (No. 12) WITH SYNTHETIC MIXTURE NO. 199: FIFTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day		Deaths in mice inoculated with undiluted culture fluid	LD ₅₀ titers determined in mice
		Uninfected	Infected		
1	3	46.2	45.9	3/6	
2	7	25.0	25.0	1/6	
3	10	25.4	28.3	2/6	
4	14	23.6	23.5	3/6	
5	17	31.8	30.9	6/6	$10^{-1.12}$
6	21	24.5	25.0	6/6	$10^{-1.88}$
7	24	33.3	32.5	6/6	$10^{-1.62}$
8	28	24.4	24.5	3/6	
9	31	31.3	33.3	6/6	
10	34	32.9	32.6	6/6	
11	38	24.5	24.6	6/6	
12	42	24.5	24.2	6/6	
13	45	29.1	31.0	4/6	
14	49	24.2	23.8	5/6	
15	53	23.9	24.1	5/6	
16	56	29.9	30.5	5/6	
17	59	29.5	29.9	2/6	
18	63	22.3	23.1	3/6	
19	66	23.2	25.9	4/6	
20	70	19.1	19.3	4/6†	
21	73	14.1	16.5	6/6	
22	77	14.7	12.5	6/6	
23	80	14.7	9.8	6/6	
24	84	13.7	7.4	5/6	
25	87	13.5	5.2	6/6	
26	90	15.6	8.7	5/6	
27	93	14.8	6.5	5/6	
28	97	11.7	2.3	4/6	
29	101	9.5	3.7	5/6	
30	105	8.1	9.2	3/6	
31	108	10.5	2.9	1/6	
32	112	6.1	1.4	5/6	
33	115	11.2	4.7	4/6	
34	119	5.0	2.2	3/6	
35	122	11.2	2.1	0/6	
36	125	8.6	0.3	2/6	
37	129	5.9	0.2	3/6	
38	133	5.6	1.2	1/6	
39	136	1.1	0.0	1/6	
40	140	8.8	1.3	3/6	

* Cultures inoculated with 0.1 ml. of the fourth culture fluid from the fourth subculture of the virus in monkey testis (No. 11), with a titer of approximately 75 LD₅₀ for mice.

† Positive infectivity test in monkey.

TABLE III
GLUCOSE UTILIZATION IN CULTURES OF MONKEY TISSUES (No. 13) WITH SYNTHETIC MIXTURE No. 199;
FOURTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day																				
		Testis			Brain			Kidney			Lung			Gut			Muscle			Diaphragm		
		Uninf.	Inf.	Uninf.	Uninf.	Inf.	Uninf.	Uninf.	Inf.	Uninf.	Inf.											
1	3	44.7	47.2	13.2	13.2	7.7	11.5	44.0	48.0	21.1	22.9	1.8	1.1	4.5	1.8							
2	7	25.0	25.0	4.6	5.3	16.1	18.9	25.0	25.0	18.2	19.5	1.8	1.6	3.4	0.8							
3	10	33.0	33.0	8.2	9.1	27.0	30.3	32.5	26.7	16.8	17.7	2.6	2.8	1.6	0.0							
4	14	24.5	24.5	6.9	10.5	24.0	24.0	24.0	24.4	13.9	16.2	3.8	2.4	3.8	1.1							
5	17	31.7	31.8	7.0	10.0	32.0	31.0	30.0	25.2	14.7	14.4	2.3	0.0	4.2	0.5							
6	21	23.4	24.2	9.3	12.5	23.6	23.6	24.0	19.4	13.7	12.8	1.3	1.1	2.7	1.1							
7	24	24.3	27.1	10.9	14.0	32.0	20.9	27.8	25.3	14.4	13.3	3.5	1.2	2.4	1.5							
8	28	19.3	22.5	9.1	14.1	23.9	14.3	24.1	18.8	14.9	11.3	4.8	2.5	4.8	3.2							
9	32	15.7	18.8	3.3	8.2	22.8	5.6	20.1	22.5	11.9	8.8	3.9	5.6	11.9	2.9							
10	35	22.8	27.0	0.0	1.5	29.9	1.9	27.5	30.3	16.1	5.2	0.0	3.1	1.6	0.0							
11	38	19.6	24.1	1.3	3.7	31.8	0.0	25.5	30.7	16.1	9.2	3.5	0.0	6.6	4.3							
12	41	16.6	21.1	1.9	4.1	28.3	0.5	27.5	28.9	13.5	6.8	1.7	4.3									
13	45	13.8	17.2	0.2	1.1	17.9	0.0	20.3	21.4	10.6	5.1	0.3	0.5									
14	48	16.9	17.1	0.3	0.0	13.0	0.8	25.3	27.1	12.0	2.4	2.9	10.7									
15	51	11.9	13.7	0.1	0.0	6.6	1.8	21.2	21.4	7.7	0.1	4.6	2.5									

			0.8	1.5	3.6	0.3	19.8	19.8	8.7	2.7	1.5	3.1
16	55	17.0	12.7	0.0	4.5	1.5	3.1	19.6	20.0	9.9	11.6	7.1
17	58	20.2	20.2	0.0	4.5	1.5	3.1					3.2
18	62	14.8	10.9			1.5	0.0	15.1	15.0	7.1	2.2	
19	65	14.0	10.4			3.3	2.6	17.1	16.4	6.6	0.2	
20	69	9.3	6.7			2.7	1.5	12.7	13.2	6.6	1.6	
21	72	9.3	3.9			0.0	0.0	8.4	11.2	7.7	1.2	
22	75	17.8	8.0					13.3	13.7			
23	78	12.0	5.4					14.1	14.1			
24	82	11.8	2.8					10.8	10.5			
25	86	9.0	4.9					8.1	9.5			
26	90	9.2	4.5					7.5	8.9			
27	93	10.0	2.2					8.3	6.7			
28	97	6.7	4.8					7.1	5.5			
29	100	7.9	4.4					12.9	7.9			
30	104	3.7	0.0									
31	107	6.4	2.3									
32	110	3.7	5.2									
33	114	3.7	2.1									
34	118	2.1	0.6									
35	121	0.0	0.0									

* Cultures inoculated with 0.1 ml. of the 19th culture fluid from the third subculture of the virus in human embryonic kidney (No. 13) with a titer of approximately 100 LD₅₀ for mice.

(C) Attempted Propagation of Lansing Virus in Cultures of Beef Embryo Tissues

It will be seen from Table V that cultures of beef embryo kidney and lung metabolized very actively and were still using all the available glucose even after 40 days of incubation; at this time, cultures of brain tissue were using

TABLE IV

INFECTIVITY TESTS IN CULTURES OF MONKEY TISSUES (No. 13) WITH SYNTHETIC MIXTURE
No. 199: FOURTH SUBCULTURE OF LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Deaths in mice inoculated with undiluted culture fluid						
		Testis	Brain	Kidney†	Lung††	Gut	Muscle	Diaphragm
1	3	4/6	5/6	2/6	2/6	0/6	2/6	1/6
2	7	0/6	0/5	2/6	0/6	1/6	3/6	0/6
3	10	0/6	4/6	2/6	5/6	3/6	0/6	0/6
4	14	1/6	1/6	6/6	6/6	5/6	0/6	0/6
5	17	4/6	4/6	5/6	5/6	5/5	4/6	0/6
6	21	3/6	3/6	6/6	6/6	3/5	4/6	0/6
7	24	6/6	2/6	6/6	6/6	6/6	4/6	0/6
8	28	4/6	0/6	6/6	5/6	3/6	0/6	0/6
9	32	6/6	0/6	6/6	5/6	3/6	2/6	0/6
10	35	5/6	0/6	4/6	3/6	1/6	0/6	0/6
11	38	4/6	0/6	2/6	5/6	4/5	1/6	0/6
12	41	2/6	0/6	3/6**	6/6	5/6	0/6	
13	45	2/6	0/6	0/6	4/6	2/6	0/6	
14	48	6/6	1/6	0/6	6/6	0/6	0/6	
15	51	4/6	0/6	1/6	4/6	0/6	0/6	
16	55	5/6	0/6	0/6	0/6	0/6	0/6	
17	58	5/5	0/6	0/6	5/6**	0/6	0/6	
18	62	6/6		0/6	4/6	3/6		
19	65	6/6		0/6	1/6	1/6		
20	69	6/6		0/6	1/6	0/5		
21	72	6/6		0/6	0/6	0/6		
22	75	3/6			2/6			
23	78	3/6			0/6			
24	82	2/6			0/6			
25	86	3/6			0/6			
26	90	4/6			2/6			
27	93	1/6			0/6			
28	97	5/6			1/6			
29	100	3/6			4/6			
30	104	2/6						
31	107	0/6						
32	110	1/6						
33	114	6/6						
34	118	2/6						
35	121	0/6						

* Cultures inoculated with 0.1 ml. of the 19th culture fluid from the third subculture of the virus in human embryonic kidney (No. 13), with a titer of approximately 100 LD_{50} .

** Positive infectivity test in monkey.

† Titration values in mice for pooled culture fluids 4, 6, and 8 were $10^{-1.30}$, $10^{-1.70}$, and $10^{-1.60}$ respectively.

†† Titration values in mice for pooled culture fluids 3, 5, and 7 were $10^{-0.12}$, $10^{-1.30}$, and $10^{-1.12}$ respectively.

only about 50% of the available glucose. Infectivity tests are summarized in Table VI, and it will be noted that there was no evidence of virus propagation in the cultures.

A second experiment in which another beef embryo was employed gave similar results.

TABLE V

GLUCOSE UTILIZATION IN CULTURES OF BEEF EMBRYO BRAIN, KIDNEY, AND LUNG WITH SYNTHETIC MIXTURE NO. 199, INOCULATED WITH LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Average % glucose used per day					
		Brain		Kidney		Lung	
		Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
1	3	15.3	28.5	47.0	49.1	44.9	48.7
2	7	15.8	15.8	24.1	22.7	20.7	22.1
3	11	14.2	17.0	23.0	24.4	22.7	22.7
4	14	20.4	30.7	33.3	33.3	33.3	33.3
5	18	23.9	22.7	25.0	25.0	25.0	25.0
6	21	22.4	26.6	31.4	30.5	32.9	32.1
7	24	30.0	20.9	31.4	30.2	30.9	21.7
8	28	18.0	18.8	24.1	23.0	23.7	23.9
9	32	18.6	16.4	23.3	25.0	25.0	25.0
10	36	18.5	13.5	24.4	24.1	23.5	24.1
11	40	16.7	12.3	25.0	24.2	25.0	25.0

* Cultures inoculated with 0.1 ml. of the fifth culture fluid from the third subculture of the virus in human embryonic kidney (No. 13), with a titer of approximately 1400 LD₅₀ for mice.

TABLE VI

INFECTIVITY TESTS IN CULTURES OF BEEF EMBRYO, BRAIN, KIDNEY, AND LUNG WITH SYNTHETIC MIXTURE NO. 199: INOCULATED WITH LANSING VIRUS*

Pooled culture fluids	Incubation (days)	Deaths in mice inoculated with undiluted culture fluid		
		Brain	Kidney	Lung
1	3	5/6	5/6	6/6
2	7	0/6	0/6	0/6
3	11	0/6	0/6	0/6
4	14	0/6	0/6	0/6
5	18	0/6	0/6	0/6
6	21	0/6	0/6	0/6
7	24	0/6	0/6	1/6
8	28	0/6	0/6	0/6
9	32	0/6	0/6	0/6
10	36	0/6	0/6	0/6
11	40	0/6	0/6	1/6

* Culture infected with 0.1 ml. of the fifth culture fluid from the third subculture of the virus in human embryonic kidney (No. 13), with a titer of approximately 1400 LD₅₀ for mice.

Level of passage in tissue culture	Mouse central nervous system					Beef embryonic brain, kidney, lung
	Human embryonic brain and cord	Human embryonic kidney	Human postnatal tonsils	Monkey testis	Monkey testis, brain, kidney, lung, gut, muscle, diaphragm	
Primary	↓ Human embryonic brain and cord	↓ Human embryonic kidney	Human postnatal tonsils	Monkey testis	Monkey testis, brain, kidney, lung, gut, muscle, diaphragm	Beef embryonic brain, kidney, lung
First subculture	↓ As above	↓ As above				
Second subculture	↓ As above	↓ As above				
Third subculture	↓ As above	↓ As above				
Fourth subculture	↓ As above	↓ As above	↓ As above	↓ As above	↓ As above	↓ As above
Fifth subculture	↓ As above		↓ As above	↓ As above		
Sixth subculture	↓ As above					

Conclusions

It has been demonstrated that Lansing poliomyelitis virus can be grown in cultures of tissues derived from monkey testis, kidney, lung, and gut. Similar experiments were carried out with cultures prepared from tissues derived from beef embryo brain, lung, and kidney. Although these tissues survived in the medium and metabolized actively, they did not support viral proliferation. These results, together with those of other investigators, point to the fact that the requirements of the Lansing virus for multiplication in tissue cultures are somewhat restricted. It would appear that more virus is produced in cultures derived from monkey testis and kidney than in cultures derived from brain.

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THE LIPIDE COMPOSITION DURING STORAGE OF HUMAN PLASMA, POOLED, IRRADIATED, AND DRIED¹

BY ELDON M. BOYD

Abstract

The purpose of this investigation was to determine the total lipide, neutral fat, total fatty acid, total cholesterol, ester cholesterol, free cholesterol, and phospholipide content, estimated by oxidative micromethods, of human plasma, pooled, irradiated, and dried, as collected by the Canadian Red Cross Blood Transfusion Service and processed at the Connaught Medical Research Laboratories. Determinations were made before and after storage as dried plasma for six months in the dark at temperatures of -40°C ., 4°C ., 20°C ., and 40°C . All lipides present in this dry powdered plasma were found to be completely taken up in the distilled water used for its reconstitution to liquid plasma. The reconstituted liquid plasma contained approximately one-half the concentration of lipides present in fresh normal human plasma, the difference being due entirely to dilution during preparation and processing, except in the instances of both free and esterified cholesterol, of which approximately one-fifth the amount present in fresh normal human plasma was found to be missing in the final product. No statistically significant loss of lipides in dried plasma occurred after storage at -40°C ., 4°C ., or 20°C .; at 40°C . there was a statistically significant loss of approximately one-fifth of practically all lipides. Ultraviolet irradiation during processing did not affect the lipide composition of dried plasma determined before and after storage at 4°C .

Introduction

The objective of the investigation to be reported below was to determine the chemical stability of lipides in human plasma, pooled, irradiated, and dried (Connaught) during its storage as dried plasma at temperatures ranging from -40°C . to $+40^{\circ}\text{C}$. The human plasma used was the standard dried plasma available in Canada from blood collected by the Canadian Red Cross Society as gifts from voluntary blood donors and pooled, irradiated, dried, and prepared for human use by the Connaught Medical Research Laboratories of the University of Toronto. Evidence of a statistically significant destruction of lipides was obtained in such dried plasma after storage for six months at 40°C . and of no statistically significant alteration in lipide content after similar storage at -40°C ., 4°C ., and 20°C .

There have been numerous reports published upon the keeping qualities of preserved human blood and blood derivatives (9, 12, 13). Nance (18) states that whole dried plasma may be transfused to a patient after storage for periods up to five years, but she makes no reference to the temperature at which such material may be safely stored. Greaves (9) reviews work upon the stability of proteins in human plasma and notes that, "Insufficient is known about the keeping qualities of different materials, with differing residual moisture contents and at different temperatures . . .". Kilduffe and DeBakey (13) state categorically that the residual water content of whole dried plasma should be 0.5% or less.

Sterile liquid plasma may be stored either at room temperature or at -30°C . without gross alteration in the amount of its albumin or globulin fractions

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Contribution from the Department of Pharmacology, Queen's University, Kingston, Ont.

according to Taylor *et al.* (20), but Lozner *et al.* (16) estimate an up to 2% loss of protein after 15 to 24 months of storage represented as an increase in plasma nonprotein nitrogen fractions. Furthermore, there are internal shifts in the globulin fraction to the advantage of the alpha fraction, according to Krejci, Sweeny, and Sanigar (14). Of the lipides in stored liquid plasma or serum, Nance (18) notes the presence of what she terms "unstable lipoids" which may account for the opaque appearance or even the formation of a sediment. This could indicate a possible hydrolysis of plasma phospholipides since it was shown by the author (3) in 1937 that opaque and milky human sera contain relatively less phospholipide in proportion to other lipides, particularly neutral fat.

According to Aylward, Mainwaring, and Wilkinson (1), there is no significant change in the amount of lipoid phosphorus in plasma or in the red blood cells of stored whole blood. There are, however, many other reported chemical alterations in stored whole blood, due largely to shifts from the cells to plasma as the concentration of glucose diminishes (12), including, for example, an increase in plasma potassium (19), inorganic phosphate (12), albumin, globulin, and fibrinogen (15), and sodium (17), with apparently no change in the concentration of plasma chloride (7).

In 1937, the author (2) reported upon the keeping qualities of human plasma lipides extracted in alcohol-ether and stored as alcohol-ether extracts either at room temperature or at 4° C. After three to six months of storage of lipides extracted from plasma, there was found to have occurred some hydrolysis of phospholipides and cholesterol esters and a slight loss of digitonin-precipitable cholesterol. Hydrolysis of phospholipides and cholesterol esters yielded higher values for residual fatty acids and hence there was an apparent increase in "neutral fat" as which the residual fatty acids are ordinarily calculated. Hydrolysis of cholesterol esters also resulted in apparent increases in the amount of free cholesterol. There were no significant alterations in the amount of total fatty acids or of total lipide. In the investigation herein reported, a similar study was made of the stability of human plasma lipides stored, however, as human plasma, pooled, irradiated, and dried (Connaught), and evidence was obtained that under these conditions of storage as dried plasma, human plasma lipides are considerably more stable than under the conditions reported previously.

Method

With the cooperation of the Canadian Red Cross Blood Transfusion Service, and the Connaught Medical Research Laboratories, dried human plasma was made available to the author in aliquots, each of which had been made from 100 ml. of pooled human plasma of lot number 141604 which had been received in Toronto for processing during August, 1951. Each aliquot had been dried, irradiated, and tested for sterility and pyrogens as in the standard technique used in preparing dried plasma for human transfusions. The particular lot used in these experiments was reported to have produced, in the pyrogen

tests, some elevation of body temperature which was within the limits adopted for plasma released for human use (8). Each dried aliquot, obtained from 100 ml. of pooled plasma, the smallest volume which could be measured with an estimated error of not over 2% in the production procedure (8), was separately dispensed in a standard distribution bottle used for drying 400 ml. of pooled plasma. Shipment of these bottles of dried plasma was received at Queen's University in September, 1951. A manufacturer's shortage of material caused delay in delivery of certain technical equipment needed in this investigation and the dried plasma was kept until January 12, 1952, when the initial extractions for lipide analyses were made.

At the latter date, the total weight of dried plasma in each bottle was determined and a sample removed and weighed in a dried weighing vessel for estimation of the residual water content by heating at 90° C. in a Fisher forced draft Isotemp oven until the sample reached a constant weight, which was usually within 24 hr. but occasionally longer. The mean \pm standard deviation residual water content of the dried plasma at this time was 3.23 ± 0.98 gm. per 100 gm. of human plasma, pooled, irradiated, and dried (Connaught). During the process of drying in the heated weighing bottles, the powdered plasma darkened perceptibly from its initial pale buff shade. From the values thus available, the dry weight in grams of powdered plasma per bottle was calculated. After a number of such calculations had been made, it became evident that there was some variation in the total weight of dried plasma per bottle and that it would be more accurate to express the lipide data as gm. per 100 gm. dry weight of plasma removed from individual container bottles. The mean \pm standard deviation dry weight of powdered plasma thus removed from 16 container bottles was 5.930 ± 0.438 gm. with a coefficient of variation of 7.4.

To determine the solubility of lipides in distilled water used to reconstitute the dried plasma, aliquots of the latter were weighed and extracted with alcohol-ether for lipide analysis, an aliquot of 400 to 500 mgm. being found by trial to contain amounts of lipides which could be determined with most convenience and accuracy by the system of lipide analysis employed. The remaining dried plasma was then reconstituted with distilled water, using a volume corresponding to the figure obtained by dividing the remaining weight by the original weight of dried plasma in the container bottle and multiplying by 100 ml. to produce reconstituted plasma similar to that used for transfusion to man. Analysis of this material revealed that it contained lipides in approximately one-half the concentration encountered in normal fresh human blood plasma. For convenience in preparing the alcohol-ether extracts, the reconstituted plasma was therefore prepared in double strength for most analyses. An aliquot of 8 to 10 ml. of the reconstituted plasma, or of 4 to 5 ml. of the double strength reconstituted plasma, was found to contain amounts of lipides satisfactory for subsequent analysis. The alcohol-ether extracts were prepared and analyzed for total lipide, neutral fat, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, and phospholipide by oxidative micromethods as previously described by the author (4).

The remaining bottles of human plasma, pooled, irradiated, and dried (Connaught) were then divided into four groups. One group was placed for storage in a custom-built Frigidaire refrigeration unit and maintained at a temperature of $-40 \pm 4^\circ\text{C}$. A second group was stored in a Kelvinator refrigeration unit at a temperature of $4 \pm 1^\circ\text{C}$. The third group was stored in the dark at room temperature of $20 \pm 4^\circ\text{C}$. The fourth group was placed in a Thelco incubator at $40 \pm 2^\circ\text{C}$. These temperatures were maintained, apart from an occasional short break due to cutoffs in Ontario Hydro power, for a period of six months, after which three bottles were removed from each group and the dried human plasma extracted and analyzed for lipides and water as described above.

Dried vs. Reconstituted Plasma Lipides

Analyses revealed that the lipides present in human plasma, pooled, irradiated, and dried (Connaught), are completely soluble in the distilled water used for reconstitution. This was demonstrated in a total of 27 comparisons of the lipide composition of dried plasma before and after reconstitution with distilled water and the mean values obtained have been assembled in Table I. There were no significant differences between values for lipides in these two groups of extracts. Subsequent lipide analyses of dried plasma were confined to extraction and analysis of reconstituted plasma with occasional checks upon extracts made before reconstitution, none of which demonstrated any failure of solution in distilled water of the lipides in dried plasma during its reconstitution.

TABLE I

THE LIPIDE COMPOSITION OF HUMAN PLASMA, POOLED, IRRADIATED, AND DRIED (CONNAUGHT) BEFORE AND AFTER RECONSTITUTION WITH DISTILLED WATER

(Values for lipides are expressed as gm. per 100 gm. dry weight of human plasma, pooled, irradiated, and dried—Connaught)

Lipide	Before reconstitution	After reconstitution
Total lipide	4.16	4.13
Neutral fat	1.35	1.25
Total fatty acids	2.63	2.59
Total cholesterol	1.01	1.02
Ester cholesterol	0.65	0.70
Free cholesterol	0.35	0.32
Phospholipide	1.35	1.40

Lipides of Reconstituted Plasma

In Table II is given a statistical summary of results obtained from lipide analyses upon 16 extracts of human plasma, pooled, irradiated, and dried (Connaught) after reconstitution with distilled water. Since it was desired to compare these data with corresponding data upon normal fresh human blood plasma and since data upon lipide content of the latter are usually

TABLE II

THE LIPIDE COMPOSITION OF HUMAN PLASMA, POOLED, IRRADIATED, AND DRIED (CONNAUGHT)
AFTER RECONSTITUTION AND BEFORE AND AFTER CORRECTIONS FOR DILUTION, COMPARED
WITH THE LIPIDE COMPOSITION OF FRESH, NORMAL, HUMAN PLASMA

(Values for lipides are expressed as mgm. per 100 ml. of plasma)

Lipide	Calculation	Reconstituted dried plasma			Fresh normal human plasma
		Direct analysis	Corrected for extraneous dilution	Corrected for maximal dilution	
Total lipide	Mean	252	462	530	530
	Standard deviation	33	61	69	74
Neutral fat	Mean	78	143	164	142
	Standard deviation	19	35	40	60
Total fatty acids	Mean	159	291	334	316
	Standard deviation	21	38	44	85
Total cholesterol	Mean	62	113	130	152
	Standard deviation	12	22	25	24
Ester cholesterol	Mean	43	78	90	106
	Standard deviation	8	15	17	25
Free cholesterol	Mean	19	35	40	46
	Standard deviation	5	10	11	8
Phospholipide	Mean	83	153	174	165
	Standard deviation	18	34	38	28

expressed as mgm. of lipide per 100 ml. of plasma, similar units were adopted in compiling Table II. The data listed in Column 3 of Table II were obtained by multiplying the analytical values, expressed as gm. lipide per 100 gm. dry weight of dried plasma, by 10 times the mean dry weight of dried plasma in the container bottles from which the extracts had been prepared. These values represent the lipide composition of human plasma, pooled, irradiated, and dried (Connaught) after reconstitution with distilled water and as ordinarily transfused into patients.

When the lipide composition of this material is compared with that of fresh blood plasma extracted and analyzed immediately after removal from healthy subjects as listed in the figures given in Column 6 of Table II, which figures are from a previous report by the author on 118 subjects using the same analytical technique (5), it may be seen that human plasma, pooled, irradiated, and dried (Connaught) contains, after reconstitution, about one-half the concentration of lipides present in fresh blood plasma of healthy man. This loss of lipides is more apparent than real, since human plasma, pooled, irradiated, and dried (Connaught) represents, after reconstitution, a considerable dilution of the original fresh human blood plasma from which it was prepared.

Similarly, the dry weight of human plasma, pooled, irradiated, and dried (Connaught) contains dry weight from substances added during its preparation, in addition to the dry weight of actual blood plasma. Hence the lipide content of the original human blood plasma remaining in human plasma, pooled, irradiated, and dried (Connaught) after reconstitution would be considerably higher than would be indicated by the values listed in Column 3 of Table II.

The dilution of fresh plasma by liquids and solids added extraneously during its collection and processing into human plasma, pooled, irradiated, and dried (Connaught) and reconstitution for human transfusion cannot be calculated absolutely because of unknown errors attached to the many steps involved in collection and processing, but an average dilution factor may be determined by assuming that the plasma pooled represented an average of the normal, healthy population. This was done with the assistance of information provided by Cull (6) upon the procedures employed by the Canadian Red Cross Blood Transfusion Service in the collection and preparation of plasma pools and by Wilson (21) upon the processing of such pools into human plasma, pooled, irradiated, and dried at the Connaught Medical Research Laboratories. From this information and using mean values for hematocrit and plasma dry weight (10) it was calculated that each 100 gm. dry weight of human plasma, pooled, irradiated, and dried (Connaught) contained 75 gm. dry weight of original plasma and 25 gm. of extraneously added dry weight. From these figures, assuming an original plasma dry weight of 8.2%, the lipide composition, in mgm. per 100 ml. of original human plasma present in dried plasma, pooled, irradiated, and dried (Connaught), was calculated by multiplying the analytical figures, in gm. of lipid per 100 gm. dry weight of human plasma, pooled, irradiated, and dried (Connaught), by the factor 109. Such values have been listed in Column 4 of Table II.

The figures listed in Column 4 of Table II may be considered as representing the mean minimal correction for dilution of plasma in human plasma, pooled, irradiated, and dried (Connaught) after reconstitution with distilled water, as will be explained below. Therefore they indicate the maximal possible loss of lipide from fresh human plasma during its processing into human plasma, pooled, irradiated, and dried (Connaught). This mean maximal loss may be calculated from values listed in Table II to have consisted of a 13% loss of total lipide due to a 26% loss of total cholesterol which in turn was due to a 26% loss of ester cholesterol and 24% loss of free cholesterol. All of these differences were statistically significant with *P* values less than 0.01 as calculated after Hill (11). None of the other differences between lipide values listed in Columns 4 and 6 of Table II was statistically significant. Thus the mean maximal alteration in plasma lipides during their collection and processing to produce human plasma, pooled, irradiated, and dried (Connaught) was loss of about one-quarter of the original amount of free cholesterol and cholesterol esters.

In addition to correction for dilution of human plasma, pooled, irradiated, and dried (Connaught) by substances added extraneously, a further correction must be made for the addition of substances from the red blood cells during the initial period when blood collected by the Canadian Red Cross Society is stored for possible immediate use as transfused whole blood. Plasma from whole blood not so used is then pooled for the preparation of dried plasma. A variable amount of diffusion of proteins and salts from erythrocytes to plasma, as discussed above in the introductory section, may occur during this period of propinquity which lasts at least two weeks, usually three weeks and occasionally longer (6). This eventually results in further dilution of the dry weight of plasma in human plasma, pooled, irradiated, and dried (Connaught). The maximal dilution possible from the erythrocytes and from addition of extraneous substances could not be greater than that which, when used in calculating the lipide content of blood plasma present in human plasma, pooled, irradiated, and dried (Connaught), would result in values for total lipide higher than those in normal fresh blood plasma. For, while the mean concentrations of neutral fat, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, and phospholipide in normal human erythrocytes are all different from corresponding mean values in normal fresh human plasma, as estimated by oxidative micromethods and as expressed in terms of mgm. per 100 ml. of either erythrocytes or plasma, the mean concentration of total lipide is the same in erythrocytes and plasma of normal man (5). Therefore even if complete hemolysis occurred in the original whole blood, the "plasma" so used to produce human plasma, pooled, irradiated, and dried (Connaught) could not contain over a mean of 530 mgm. of total lipide per 100 ml.

To correct for the maximal possible dilution of plasma and thus obtain values for the maximal possible lipide content of plasma remaining in human plasma, pooled, irradiated, and dried (Connaught), the figures listed in Column 3 of Table II were multiplied by the factor 2.1 to obtain the figures listed in Column 5 of Table II. When these figures are compared with those listed in Column 6, it may be calculated that there remained a statistically significant difference averaging 14% decline in the concentration of total cholesterol, averaging 15% decline in that of ester cholesterol and a difference of limited statistical significance, averaging 13% decline, in the concentration of free cholesterol. No other differences were statistically significant after the criteria of Hill (11).

Thus it may be concluded that in the collection of fresh human plasma by the Canadian Red Cross Blood Transfusion Service and its processing by the Connaught Medical Research Laboratories into human plasma, pooled, irradiated, and dried, there is lost 15 to 26% of the original plasma cholesterol esters and 13 to 24% of the original free cholesterol and that there is no other loss of plasma lipides. The low values for lipides in human plasma, pooled, irradiated, and dried (Connaught) after reconstitution are due almost entirely to a dilution from internal and external sources of the original fresh human plasma of from 1 in 1.8 to 1 in 2.1.

Storage of Dried Plasma

The effects of storage for six months at varying temperatures upon the lipide composition of human plasma, pooled, irradiated, and dried (Connaught) are summarized statistically in Table III. The prestorage figures listed in Column 3 of Table III are means and standard deviations from the analysis of 16 different extracts, while the averages in Columns 4 to 7 are each of lipide analyses upon six different extracts, representing duplicates upon each of three samples of human plasma, pooled, irradiated, and dried (Connaught). No destruction or loss of lipides occurred during six months of storage at temperatures of -40° C. or 4° C. ; there was a statistically significant loss of most lipides after storage for six months at 40° C. ; there was some loss of some lipides of equivocal statistical significance after storage for six months at 20° C.

TABLE III

THE EFFECT OF STORAGE FOR SIX MONTHS AT VARYING TEMPERATURES UPON THE LIPIDE COMPOSITION OF HUMAN PLASMA, POOLED, IRRADIATED, AND DRIED (CONNAUGHT)

(Values for lipides are expressed as gm. per 100 gm. dry weight of human plasma, pooled, irradiated, and dried—Connaught)

Lipide	Calculation	Before storage	After storage at			
			-40° C.	4° C.	20° C.	40° C.
Total lipide	Mean	4.24	4.33	4.38	3.94	3.34
	Standard deviation	0.56	0.51	0.20	0.13	0.14
Neutral fat	Mean	1.31	1.27	1.34	1.10	0.97
	Standard deviation	0.32	0.14	0.28	0.15	0.15
Total fatty acids	Mean	2.67	2.68	2.76	2.44	2.09
	Standard deviation	0.35	0.37	0.13	0.14	0.14
Total cholesterol	Mean	1.04	1.06	1.08	0.96	0.81
	Standard deviation	0.20	0.08	0.06	0.03	0.03
Ester cholesterol	Mean	0.72	0.70	0.76	0.66	0.56
	Standard deviation	0.14	0.10	0.07	0.15	0.02
Free cholesterol	Mean	0.32	0.36	0.32	0.30	0.25
	Standard deviation	0.09	0.02	0.03	0.03	0.01
Phospholipide	Mean	1.40	1.52	1.44	1.43	1.20
	Standard deviation	0.31	0.26	0.37	0.08	0.08

Storage at 40° C. resulted in a statistically significant loss of all lipides (P less than 0.01) except of phospholipides from the prestorage levels. The mean destruction of digitonin-precipitable free and ester cholesterol was 22%, of neutral fat 26%, and the equivocal loss of phospholipides averaged 14%. These changes produced a mean loss of 22% in total cholesterol and total fatty acids and of 21% in total lipide. The bottled dried plasma was found to contain an average of 5.18% water in this batch stored at 40° C. which might

suggest that hydrolysis could account for the loss of cholesterol esters and phospholipides. Such hydrolysis would have produced an increase in free cholesterol and in residual fatty acids which are calculated as neutral fat and these changes did not occur.

Storage at either -40°C . or at 4°C . did not affect the lipide composition of human plasma, pooled, irradiated, and dried (Connaught). The only difference noted between batches stored at these two temperatures was in water content, which averaged 6.45% in the material stored at -40°C ., while that stored at 4°C . averaged 5.32%. While the difference was statistically significant, the calculations were based upon data from only three bottles of material in each case.

Storage at 20°C ., or average room temperature in this department at Queen's University in Kingston, resulted in a small decrease in the mean concentration of several lipides which averaged 9% and was calculated to have no statistical significance. Viewed in line with the significant changes at 40°C ., there was possibly some minor destruction of some lipides even at 20°C .. Uptake of water by human plasma, pooled, irradiated, and dried (Connaught) after six months of storage was least, on the average, at this temperature, the mean value for water content being 4.98%.

Ultraviolet Irradiation

During its processing, human plasma, pooled, irradiated, and dried (Connaught) is irradiated with ultraviolet light with the object of minimizing the possibility of transmission of serum jaundice (8). This is done after pooling plasma received at the Connaught Medical Research Laboratories from the Canadian Red Cross Blood Transfusion Service (21); and plasma is also exposed to germicidal ultraviolet irradiation when pooled from time-expired unused whole blood by the Canadian Red Cross Blood Transfusion Service.

TABLE IV

THE LIPIDE COMPOSITION OF HUMAN PLASMA, POOLED, IRRADIATED, AND DRIED (CONNAUGHT) COMPARED WITH THAT OF HUMAN PLASMA, POOLED, NOT IRRADIATED, AND DRIED (CONNAUGHT)

(Values for lipides are expressed as gm. per 100 gm. dry weight of human plasma, pooled, irradiated or not irradiated, and dried (Connaught))

Lipide	Before storage		After storage at 4°C .	
	Irradiated	Not irradiated	Irradiated	Not irradiated
Total lipide	4.24	4.37	4.38	4.22
Neutral fat	1.31	1.55	1.34	1.39
Total fatty acids	2.67	2.83	2.76	2.68
Total cholesterol	1.04	1.01	1.08	1.05
Ester cholesterol	0.72	0.63	0.76	0.74
Free cholesterol	0.32	0.38	0.32	0.31
Phospholipide	1.40	1.39	1.44	1.27

Through the courtesy of Dr. A. M. Fisher and at the request of the author, several bottles of lot number 141604 of human plasma, pooled, irradiated, and dried (Connaught) were prepared without the customary irradiation at the Connaught Medical Research Laboratories. These were extracted and analyzed for lipides in the same manner and at the same time as the standard irradiated material, before and after storage at 4° C. for six months, in sextuplicate extracts of material from each bottle. The results are listed in Table IV. No significant differences were noted between the lipide content of irradiated and nonirradiated material. The water content of the non-irradiated material was 2.03% before storage and 4.68% after storage, both values being lower than the lowest water content of irradiated material at corresponding times but based upon single determinations in each case of the nonirradiated material.

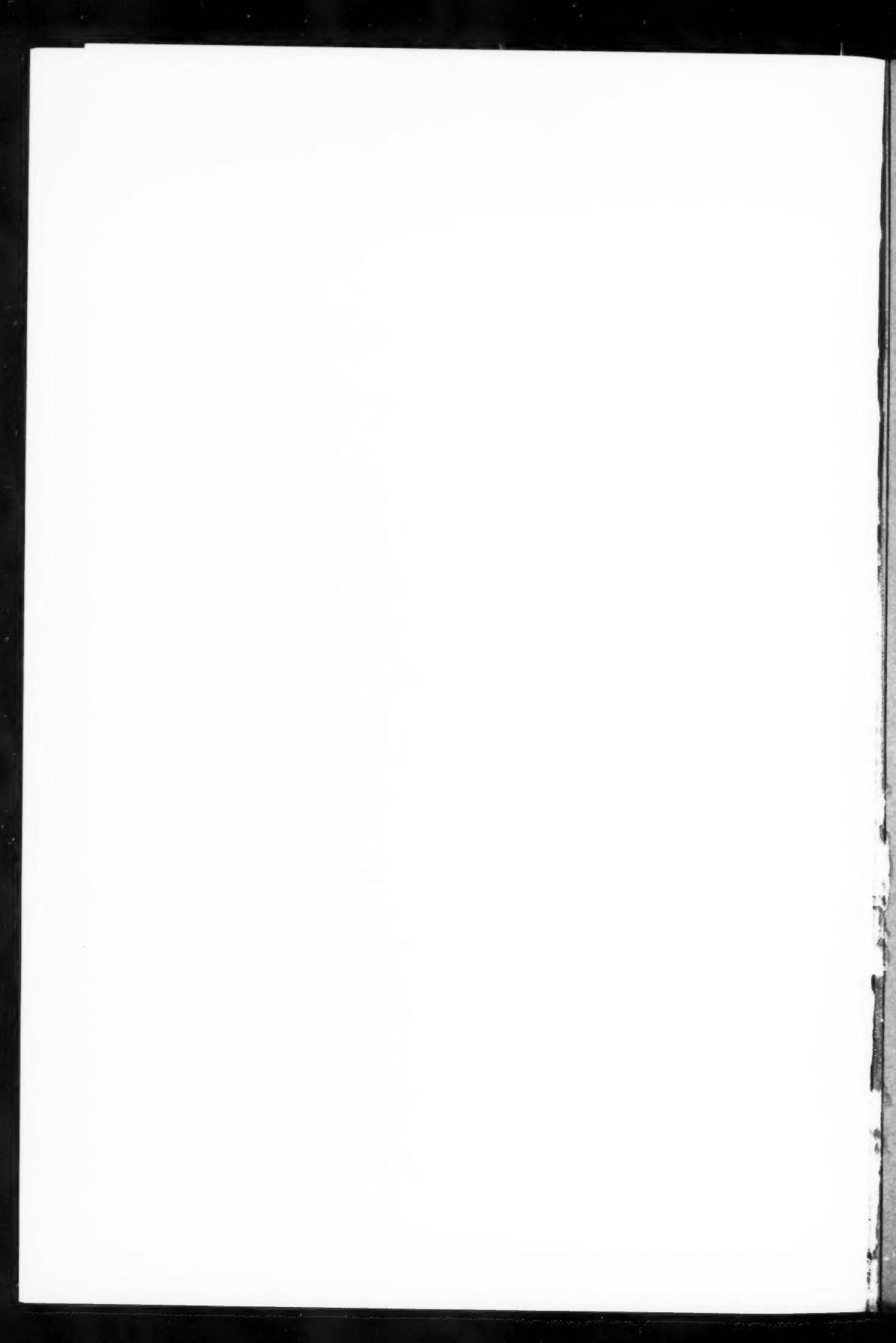
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